MECHANISMS AND FUNCTIONAL ROLES OF NUCLEAR RESPIRATORY FACTOR 1 (NRF1) BINDING SITES IN THE HUMAN GENOME

by

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MECHANISMS AND FUNCTIONAL ROLES OF NUCLEAR RESPIRATORY FACTOR 1 (NRF1) BINDING SITES IN THE HUMAN GENOME

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Genome-wide studies have suggested that NRF1 regulates transcription of ~5-6% of human genes, including nuclear genes encoding mitochondrial products. My thesis focus is in neural systems in which NRF1 is a master regulator.

Prader-Willi syndrome (PWS) results from genetic loss of function of an imprinted domain in human chromosome 15q11.2. I confirmed NRF1 regulation of ~83% of PWS-region genes using chromatin immunoprecipitation (ChIP). Further studies focused on evolution of this region. Uniquely in marsupials, *SNRPN* and the ancestral *SNRPB'* gene are adjacent each with an intronic snoRNA paralog. Based on molecular phylogenetics, a model is proposed for origin of each PWS snoRNA from a single ancestral snoRNA. Thus, most extant eutherian PWS genes originated by stepwise duplication and divergence over the past ~180 million years.

Circadian rhythms regulate organismal physiology in a 24 hour day-night cycle. Functional NRF1 binding sites in promoters/enhancers were found for ~56% of circadian regulatory genes using bioinformatics, ChIP, *NRF1* siRNA assays, and luciferase reporter constructs having significantly reduced transcriptional activity on mutation of NRF1 sites. Further, co-immunoprecipitation showed that NRF1 and the phosphorylated, active form of CLOCK interact in a molecular complex. In serum-induced NIH3T3 cells with circadian oscillations of *Dbp* and *Nr1d1* mRNA, *Nrf1* mRNA and protein levels show ultradian

oscillations. Hence, NRF1 regulates numerous circadian regulatory genes and interacts with CLOCK, suggesting multiple roles in circadian biology.

Additional studies included finding that NRF1 regulates ~45% known hereditary spastic paraplegia (HSP) genes, that NRF1 activates its own transcription, and that the number of NRF1 sites determine the degree of transcriptional activation.

In summary, NRF1 is a master regulator in PWS, circadian rhythms, and HSP. Identification of NRF1 target genes and mechanisms will lead to an understanding of the evolution, functions, disease processes, and therapeutic targets within gene regulatory networks involving NRF1. Circadian rhythms are disrupted by travel, shift-work, and in illness, including infection, psychiatric and sleep disorders, obesity, diabetes, and cancer. Consequently, understanding body clocks will provide insights into the pathogenesis of these disorders and potentially lead to improved treatment and prevention options, which will have enormous public health impact.

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PREFACE

Foremost, I would like to show my earnest appreciation to my mentor, Dr. Robert D. Nicholls, for his continuous encouragement in my research work, his constructive guidance in writing and his intelligence that impresses me in so many ways. I would like to express my sincere gratitude to Dr. Eleanor Feingold, Dr. Robert Ferrell, Dr. Susanne Gollin and Dr. Panayiotis (Takis) Benos, who serve as my committee members and give comments to my thesis.

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Last but not least, I would like to express the deepest love to my parents and my husband. Without their support and encouragement, I would not be able to accomplish this work. I want to dedicate my thesis to my dearest daughter, Annabelle, who brings so much happiness and new meaning to my life!

Abbreviations:

AS	Angelman syndrome
ChIP	Chromatin immunoprecipitation
ChIP-chip	Chromatin immunoprecipitation on chip
CREB1	cAMP responsive element binding protein 1
C_T	Threshold cycle
DMR	Differentially methylated region
ER	Endoplasmic reticulum
ESRRA	estrogen-related receptor alpha, also known as ERRα
GABP	GA binding protein transcription factor, alpha subunit
GRN	Gene regulatory network
HSP	Hereditary spastic paraplegias
IC	Imprinting center
lincRNA	Long intergenic noncoding RNA
miRNA	microRNA
NRF1	Nuclear respiratory factor 1
PPARs	Peroxisome proliferator-activated receptors
PWS	Prader-Willi syndrome
PyPu	Pyrimidine and purine
QRT-PCR	Quantitative real-time PCR
R	Adenine or guanine
RCR	Regional control region

snoRNAs	Small nucleolar RNAs
TF	Transcription factor
TFAM	Mitochondrial transcription factor A
TSS	Transcription start site
WT	wildtype
YY1	Yin Yang 1
Y	Cytosine or thymine (uracil)
ΔG	Gibbs energy (thermodynamics)

1.0 INTRODUCTION

1.1 NUCLEAR RESPIRATORY FACTOR 1

1.1.1 Gene and protein

Nuclear respiratory factor 1 (NRF1, also originally named α-Pal) is a highly conserved, phosphorylated nuclear protein (**Figure 1.1**). It is encoded by a gene with 11 exons including a non-coding first exon. NRF1 was first found to regulate genes in mitochondrial respiratory function (Scarpulla, 2008). Subsequent research has found that NRF1 recognition sites occur in many nuclear genes encoding factors involved in mitochondrial function and biogenesis (Evans & Scarpullar, 1989; 1990; Virbasius *et al*., 1993; Scarpulla, 2008; Kelly & Scarpulla, 2004), cell cycle regulation (Cam *et al*., 2004; Efiok & Safer, 2000), and neuronal functions (Smith *et al*., 2004; Chang *et al*., 2005). As a transcription factor (TF), NRF1 forms homodimers and recognizes a unique DNA binding sequence, YGCGCAYGCGCR, (Scarpulla, 2006). This motif consists of a palindromic, direct repeat, and $(PyPu)_n$ sequence structure, but the relative contributions of each of these alternative components to binding affinity are not known. In virtually all described circumstances, NRF1 functions as a positive TF to upregulate gene expression (Scarpulla, 2008), although binding of chicken NRF1 at the transcription start site (TSS) of the histone H5 gene inhibits transcription (Gómez-Cuadrado *et al*., 1995), as may regulation by the sea urchin NRF1

(P3A2) at the *CyIIIa* gene (Bogarad *et al*., 1998). The regulatory roles of NRF1 may thus be complex and depend on the context of the regulatory elements.

NRF1 has an ancient origin in animals, with orthologs identified in mouse (Nrf1), chicken (NRF1) (Gómez-Cuadrado *et al*., 1995), zebrafish (nrf1) (Becker *et al*., 1998), sea urchin (P3A2) (Calzone *et al*., 1991; Höög *et al*., 1991), and *Drosophila* (EWG) (DeSimone & White, 1993), all that share a highly conserved DNA binding region (**Figure 1.1**). Recent molecular phylogenetic studies have identified NRF1 orthologs in all animals with whole genome sequences except for the worm *C. elegans* (R.D. Nicholls unpublished data). The DNA binding domain of NRF1 is unique and not shared with any other TF class. The constrained amino acid sequence of the DNA binding domain of NRF1 suggests that a similar binding site is recognized across the animal kingdom. Indeed, *Drosophila* EWG binds to an identical DNA motif as mammalian NRF1 (Fazio *et al*., 2001).

Knock-out and mutation studies in animals indicate that NRF1 has irreplaceable functions in animal development and differentiation. In mouse, a homozygous *Nrf1* knockout mutation is lethal between embryonic days E3.5 and E6.5 (Huo & Scarpulla, 2001). Although *Nrf1* -/- blastocysts appear normal in morphology, these could not grow *in vitro* in cell culture and had significantly reduced amounts of mitochondrial DNA as well as decreased mitochondrial staining with a fluorescent dye that measures mitochondrial membrane potential (Huo & Scarpulla, 2001). Disruption of zebrafish *nrf1* is lethal at the larval stage and also shows a specific retinal phenotype with loss of all photoreceptor neurons and their precursors (Becker *et al*., 1998). Similarly, loss of the sea urchin P3A2 is embryonic lethal (Bogarad *et al*., 1998). Partial loss-of-function mutations of *EWG* in fly show severe neuronal and muscle defects while complete loss-of-function mutations are embryonic lethal (Fleming *et al*., 1989; DeSimone & White, 1993; Haussmann *et al*., 2008).

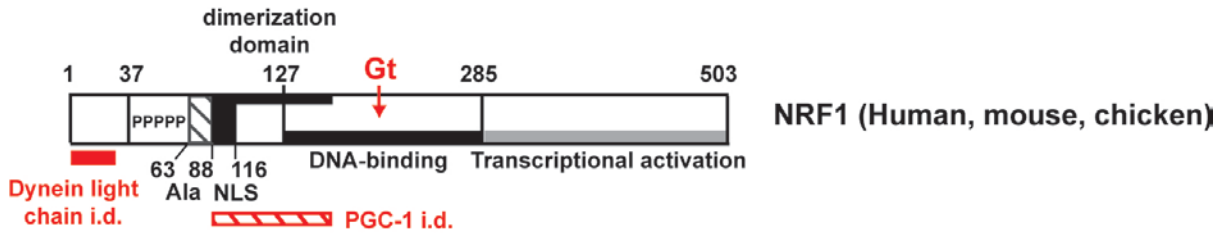


Figure 1.1. Structure of the NRF1 polypeptide. NRF1 from human, mouse, and chicken are 99-100% identical over the entire sequence, compared with the orthologs (not shown) from sea urchin (P3A2; 67.5% identity in the DNA binding domain) and *Drosophila* (EWG; 59.7% identity in the DNA binding domain). The position of polypeptide structural domains are indicated, including alanine (Ala)-rich (hatched box), nuclear localization signal (NLS, black vertical box), DNA binding (black horizontal box), dimerization (small black horizontal bar) and transcriptional activation (grey box) domains. NRF1 has 5 serine residues that are phosphorylated (P), and four of these serines are conserved in P3A2 and EWG. Other symbols are: interaction domains (i.d.) for dynein light chain and PPARGC1A/PRC; Gt: position of the RRF126 gene-trap β-geo fusion truncating the mouse Nrf1 polypeptide. Modified from: Höög *et al*., 1991; Efiok *et al*., 1994; Gómez-Cuadrado *et al*., 1995; Gugneja *et al*., 1996; Gugneja *et al*., 1997; Herzig *et al*., 2000; Andersson & Scarpulla, 2001; Huo & Scarpulla, 2001; Nicholls RD, unpublished data.

1.1.2 NRF1 and mitochondria

The mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells. Known as "cellular power plants", mitochondria produce energy, adenosine triphosphate (ATP), which is used as a major source of chemical energy. Mitochondria are also involved in pyruvate and fatty acid oxidation, nitrogen metabolism, and oxidative phosphorylation (Scarpulla, 2008). A unique feature of mitochondria is that they contain their own genetic system, mitochondrial DNA (mtDNA), which encodes a number of gene products that contribute to the structure and function of the organelle (Scarpulla, 2008). Although the mitochondrion has its own genetic information, the limited capacity of mtDNA requires nuclear genes to provide major respiratory subunits and to support mtDNA transcription and replication (Gopalakrishnan & Scarpulla, 1995). These nuclear genes encode most of the structural and catalytic components involved in the electron transport chain and oxidative phosphorylation system, as well as genes that control mitochondrial transcription, translation and DNA replication (Scarpulla, 2008). NRF1 is one of several key TFs that regulate transcription of nuclear genes encoding mitochondrial products. Other key TFs in nuclear control of mitochondrial biogenesis include GABP (GA binding protein transcription factor, alpha subunit, also known as NRF2), ESRRA (estrogen-related receptor alpha, also known as ERRα), PPARs (peroxisome proliferator-activated receptors), CREB1 (cAMP responsive element binding protein 1, also known as CREB), Sp1 and YY1 (Yin Yang 1, Scarpulla, 2008). As noted above, NRF1 was first identified and cloned in studies on transcriptional regulation of cytochrome *c* and cytochrome oxidase subunit genes (Evans & Scarpulla, 1989; Gopalakrishnan & Scarpulla, 1994; Virbasius *et al*., 1993). Bioinformatics analysis and chromatin immunoprecipitation (ChIP) using cancer cell lines showed that NRF1 binds to the promoters of many nuclear genes that are

involved in mitochondrial biogenesis and regulates transcription of these genes, including *TFAM* (mitochondrial transcription factor A), and others (Cam *et al*., 2004; Scarpulla, 2006).

Mitochondrial function is hypothesized to be associated with exercise and aging, NRF1 and its coactivators (e.g., PPARGC1A, also known as PGC-1α) have also been studied in these important physical conditions (Scarpulla, 2008). Under the normal situation in some cell types, NRF1 function involves co-activation by PPARGC1A to recruit RNA polymerase to promoters of nuclear target genes. It has been shown that exercise induces an increase in PPARGC1A and NRF1 in skeletal muscle as well as mitochondrial biogenesis (Murakami *et al*., 1998; Baar *et al*., 2002). In aging, reports have shown PPARGC1A and NRF1 expression levels are different comparing young versus aged subjects (Lezza *et al*., 2001, Viña *et al*., 2009), suggesting changes in mitochondrial biogenesis, but the answers are not yet definitive and the mechanism underneath is still obscure.

Studies of the function of NRF1, its coactivators, and other interacting TFs and regulators will help us better understand the mechanism of mitochondrial biogenesis, oxidative stress and aging, as well as how physical exercise can improve the activity of mitochondria to prevent its impairment by aging.

1.1.3 NRF1 in neuronal development

Although most of the early studies on NRF1 focused on mitochondrial functions, it is becoming increasingly clear that NRF1 not only regulates nuclear genes in mitochondrial biogenesis but also regulates numerous genes in neuronal and other systems.

For example, NRF1 was found to up-regulate transcription of the human *IAP* (Intergrin-associated protein or CD47) gene, involved with processes of memory formation in the rat hippocampus and regulation of dendritic outgrowth and synaptic transmission in developing

cortical neurons through the activation of MAPK (Huang, *et al*., 1998; Chang & Huang, 2004; Numakawa, *et al*., 2004). Subsequently, NRF1 was found to increase neurite outgrowth in part through the regulation of *IAP* (Chang, *et al*., 2005). NRF1 is also important for expression of *FMR1*, the fragile X mental retardation syndrome gene (Smith *et al*., 2004). More recent studies showed that NRF1 regulates gene expression of Synapsin I, a phosphoprotein that plays a role in regulation of axonogenesis and synaptogenesis (Wang *et al*., 2009), as well as several NMDA receptor (NR) genes which play important roles in neuronal plasticity and synaptic transmission associated neuronal functions (Dhar & Wong-Riley, 2009). The same group also investigated NRF1 regulation of cytochrome *c* oxidase (*COX*) subunits (Dhar *et al*., 2008; Dhar *et al*., 2009) to perhaps provide a link between energy generation and synaptic transmission. Additionally, studies of the NRF1 ortholog in zebrafish showed that loss of function led to a specific loss of photoreceptor neurons and their precursors (Becker *et al*., 1998) and expression of EWG, the *Drosophila* ortholog, is neuronal specific (Haussmann *et al*., 2008).

The above experimental data implies that NRF1 may play additional roles in neuronal systems and could be involved in neural-related disease pathways. My major focus has been to identify which systems are regulated by NRF1 and the mechanisms of action by NRF1.

1.1.4 Genome-wide studies of NRF1 binding sites

Transcription is initiated or repressed by the assembly of TFs at their binding sites in *cis*-acting DNA elements, usually in the promoter region of a gene, with the TSS defining where the gene begins to be transcribed into RNA (Wasserman & Sandelin, 2004). Besides promoters, other *cis*-acting DNA elements include enhancers (activation elements), silencers (repression elements) and insulator (boundary elements, Villard, 2004; Riethoven, 2010). These elements can be hundreds

or thousands of base pairs from 5' or 3' of the TSS (Villard, 2004; Riethoven, 2010). According to their function, TFs can most simply be divided into two groups. One group is the basal TFs that are necessary for transcription to occur, e.g., RNA polymerase II, and the other group includes the gene specific TFs which promote (as an activator) or suppress (as a repressor) basal transcription (Villard, 2004). Studying the exact mechanisms of how a TF regulates gene expression will be helpful in understanding gene functions and gene regulatory networks (GRNs), and how these operate in normal development as well as the roles in disease.

One of the approaches to understand the functions of a TF is to identify the target genes and regulated gene networks. In finding such a GRN, one can use the data to analyze TF function in certain diseases and identify new genes and mechanisms in disease regulation. In order to search for candidate TF binding sites in the genome, a suitable first step in the era of genome sequences is a computational approach for specific binding site sequences to identify candidate genes. Next, several genome-wide experimental methods have been established to prove functional TF binding sites: 1) microarray analysis, e.g., Chromatin immunoprecipitation on chip (ChIP-chip); 2) gene silencing by RNA interference (siRNA) or TF overexpression (a complementary approach to siRNA); and 3) next generation sequencing, e.g., ChIP-seq and RNA-seq (Goutsias & Lee, 2007; MacQuarrie *et al*., 2011; Pareek *et al*., 2011). With these approaches, a list of target genes will be identified and can be focused on for more in depth functional studies. To date, many experimental methods, including *in vitro* assays such as electrophoretic mobility shift assay (EMSA) (Fried & Crothers, 1981), reporter assays in cell culture, and *in vivo* methods such as ChIP (Kuo & Allis, 1999) have been applied to elucidate the interaction of a TF and DNA sequences. Nevertheless, these studies can only be performed when the location of regulatory element is known or predicted. In large and complex genomes, discovery of *bona fide* TF binding sites are challenging due to

factors such as 1) poor definition of gene promoters; 2) the distance between TF binding sites (e.g., in enhancers) and promoters can be 1 kb or more and up to a megabase; 3) the degeneracy in TF recognition motifs around a consensus nucleotide sequence; and 4) the variety of mechanisms, such as spacing and arrangement of TF binding sites, that are involved in transcriptional regulation (Hallikas *et al*., 2006; Villard, 2004).

Recent approaches have used computational bioinformatics to develop algorithms capable of searching genome sequences for potential TSS and TF binding sites. Typically, several classes of computational programs have been applied to the search for potential TF binding motifs in a genome and each has strengths and weaknesses (Wasserman & Sandelin, 2004). With the availability of representative mammalian genome sequences, comparative genomics provides a powerful tool to systematically discover functional elements in a genome (Wasserman *et al*., 2000; Dubchak *et al*., 2000; Xie *et al*., 2005). In particular, the NRF1 motif has been found to be the most highly conserved putative TF binding motif in the human genome (Xie *et al.*, 2005). The basis for this is likely to have several explanations including 1) NRF1 has a longer recognition motif than most TFs; 2) the NRF1 binding site allows less degeneracy than most TF binding sites which can be highly degenerate; and 3) NRF1 gene regulation, once established by evolutionary processes, may be associated with stronger selective pressure and/or reduced compensatory changes in TF regulation than for other TF-target gene interactions. The first two of these explanations are involved while the third is a hypothesis that remains to be tested. Experimentally, these considerations provide a strong rationale for inclusion of bioinformatics studies to identify and understand NRF1 functions in GRNs.

To date, genome-wide studies in human by ChIP-chip found NRF1 binding sites in 5.3% of ~13,000 gene promoters (Cam *et al*., 2004). Nevertheless, this study used a karyotypically highly

rearranged cancer cell line, and thus many epigenetically regulated or tissue-specific target genes would not have been identified (including virtually all the target genes identified in this thesis). A strictly bioinformatics study examining the distribution of 8-mer nucleotide sequences (FitzGerald *et al.*, 2004) found that a sequence (CGCVTGCG) similar to NRF1 binding sites was found in 6% of the 13,010 promoters analyzed, although the result may be slightly overestimated since the core of the consensus NRF1 binding site is longer (GCGCANGCGC). Indeed, a GCGCatGCGC motif was found in 5.3% of 10,577 promoters (Xi *et al*., 2007). Using comparative analysis of the human, mouse, rat and dog genome sequences to identify conserved regulatory motifs in promoters of ~ 17,700 genes, Xie *et al.* (2005) found 6.1% had conserved NRF1 binding sites. These studies provide a preliminary estimate of the frequency of NRF1 target genes in the human genome, although as no experimental confirmation was done it is unknown what percentage of identified sites are functionally relevant. Furthermore, none of these studies examined non-promoter sequences, so the potential contribution of NRF1 to long-range gene regulation is completely unknown.

1.2 PRADER-WILLI SYNDROME

1.2.1 Clinical phenotype and diagnosis

Prader-Willi Syndrome (PWS) is a complex congenital disorder that involves genetic abnormalities in chromosome 15q11-q13, with an incidence of ~1:15,000-1:25:000 births (Holm *et al*., 1993; Buiting, 2010; Wattendorf & Muenke, 2005). It is the most common form of obesity caused by a genetic syndrome (Wattendorf & Muenke, 2005). The clinical phenotype of PWS includes severe hypotonia, respiratory distress, feeding difficulties and failure-to-thrive in early infancy, followed by hyperphagia and development of obesity in later infancy or early childhood (Holm *et al*., 1993; Nicholls & Knepper, 2001; Gunay-Aygun *et al*., 2001). Patients are delayed in early motor and language development, as well as having some degree of cognitive impairment and hypogonadism (Holm *et al*., 1993; Gunay-Aygun *et al*., 2001). Other common features include short stature, characteristic facial features, strabismus and scoliosis (Holm *et al*., 1993; Wattendorf & Muenke, 2005). Patients also have common behavioral abnormalities, e.g., temper tantrums, stubbornness, manipulative behavior and obsessive-compulsive characteristics (Holm *et al*., 1993; Nicholls & Knepper, 2001). A prevalence rate of 25% for non-insulin-dependent diabetes mellitus was found in PWS adults due to obesity (Butler *et al*., 2002).

1.2.2 Genetics and epigenetics

At the molecular level, genes within the 2 Mb PWS region are regulated by the process of genomic imprinting. Genomic imprinting is an epigenetic mechanism leading to uniparental expression of certain genes in mammals and is established by a genetic element called the imprinting center (IC) during gametic and early preimplantation development (Nicholls & Knepper, 2001; Morison *et al*., 2005). The most common cause of PWS is a 5-6 Mb *de novo* deletion exclusively on the paternally-inherited allele. There are two classes of deletions in PWS patients, with two proximal breakpoints 1 and 2 (BP1, BP2) in 15q11 and a single distal BP3 in 15q13. PWS patients can also have maternal uniparental disomy (UPD) of chromosome 15, or in rare cases imprinting defects generating a maternal imprint on the paternally-derived chromosome (Nicholls & Knepper, 2001; Horsthemke & Buiting, 2006). In each circumstance, PWS arises from the functional loss of a set of paternally-expressed genes.

To date, at least 13 paternally-expressed genetic loci have been identified in the PWS-region in human, six of which encode polypeptides, including *MKRN3* (Jong *et al*., 1999a; 1999b), *MAGEL2* (Boccaccio *et al*., 1999; Lee *et al*., 2000), *NDN* (Jay *et al*., 1997; MacDonald & Wevrick 1997), *C15orf2* (Buiting *et al*., 2007), and the bicistronic *SNURF-SNRPN* [Gray *et al*., 1999a, **Figure 1.2(a)**]. A second imprinted disorder, Angelman syndrome (AS), is a neurological disease associated with genetic mechanisms that lead to a loss of function of a maternally-expressed imprinted gene, *UBE3A*, located telomeric to the PWS domain [**Figure 1.2(a)**]. Most PWS genes have an exclusive or predominant expression pattern in neurons (Cavaillé *et al*., 2000; Nicholls & Knepper, 2001; Runte *et al*., 2001), and their functions are only partially understood. The bicistronic locus *SNURF-SNRPN* encodes two polypeptides. *SNRPN* encodes a brain-specific

spliceosome protein, SmN, which replaces the otherwise constitutive SmB'/B protein during late fetal and postnatal brain development (Sharpe *et al*., 1990, McAllister *et al*., 1989), while the upstream *SNURF* encodes an independent nuclear protein of unknown function (Gray *et al*., 1999a). *SNURF-SNRPN* is also the host gene of five classes of box C/D small nucleolar RNAs (snoRNAs). The snoRNAs include *SNORD107* (previously *HBII-436*, 1 copy), *SNORD64* (previously *HBII-13*, 1 copy), *SNORD109A* (previously *HBII-438A*, 1 copy), *SNORD116* (previously *HBII-85*, 29 copies), *SNORD115* (previously *HBII-52*, 47 copies) and *SNORD109B* (previously *HBI-438B*, 1 copy, Cavaillé *et al*., 2000; Runte *et al*., 2001; Buiting, 2010; Zhu, *et al*., in preparation), most of which have unclear function and targets, except that *SNORD115* has been identified to regulate serotonin receptor 2C mRNA splicing (Kishore & Stamn, 2006).

The complex genetic locus encoding the polycistronic *SNURF-SNRPN*-snoRNA loci appears to play a major role in PWS, based on the location of the imprinting regulatory region at its 5' end [**Figure 1.2(a)**; Nicholls & Knepper 2001; Horsthemke & Buiting 2006], its disruption by two classes of balanced translocations (Wirth *et al*., 2001), and the finding that mice with a deletion of *Snurf-Snrpn* and the snoRNA clusters have a "partial mouse PWS" phenotype (Tsai *et al*. 1999). More recent studies showed that NECDIN (a MAGE-family protein, encoded by *NDN*) and MAGEL2 bind to several proteins in the region of the centrosome, and that axonal outgrowth is abnormal in *Ndn*-deficient embryos (Lee *et al*., 2005), while other studies suggest that in preadipocytes NECDIN inhibits a TF, E2F4, to prevent adipogenesis which is consistent with its long-postulated role in post-mitotic cells (Tseng *et al*., 2005). NECDIN also appears to play a role in the neuronal function of respiration (Ren *et al*., 2003; Zanella *et al*., 2008). *Magel2* is a clock-controlled circadian output gene (Kozlov *et al*., 2007). All PWS genes have similar expression patterns with highest or exclusive expression in neurons, especially during development (Cavaillé *et*

al. 2000; Runte et al., 2001; Lee *et al*., 2003; Watrin *et al*., 2005), and a common regulatory element specific for neurons has been proposed (Watrin *et al*., 2005).

The PWS region has an intriguing evolutionary history in mammals and shows clear evidence of relatively rapid and recent evolutionary change. *SNRPN* and the PWS region snoRNAs derive from an ancestral duplication of a *SNRPB'* gene in a therian ancestor (Gray *et al*., 1999; Rapkins *et al*., 2006; unpublished data). *Mrkn3*, *Magel2*, *Ndn*, and *Frat3* in rodents are all intronless genes and acquired by retrotranposition events in a eutherian or rodent ancestor, respectively (Jong *et al*., 1999a; 1999b; Gray *et al*., 2000, 2001; Chai *et al*., 2001). *C15orf2* shows a predominantly testis-specific expression pattern (Färber *et al*., 2000), with low expression in fetal brain and originates from a retrovirus in an ancestral primate (Buiting *et al*., 2007). In mouse, the homologous PWS region is in chromosome 7C [**Figure 1.2(b)**], except that mouse doesn't have *C15orf2* and *PWRN1* (Buiting *et al*., 2007), but gained *Frat3* by retrotransposition (Chai *et al*., 2001). The rodent locus also does not have *Snord109* copies and has a rodent specific microRNA (miRNA), miR-344 [**Figure 1.2(b)**]. Loss of imprinted gene expression in mouse 7C leads to a similar but more severe neonatal phenotype as human (Nicholls & Knepper, 2001; Stefan *et al*., 2005; Stefan *et al*., in preparation).

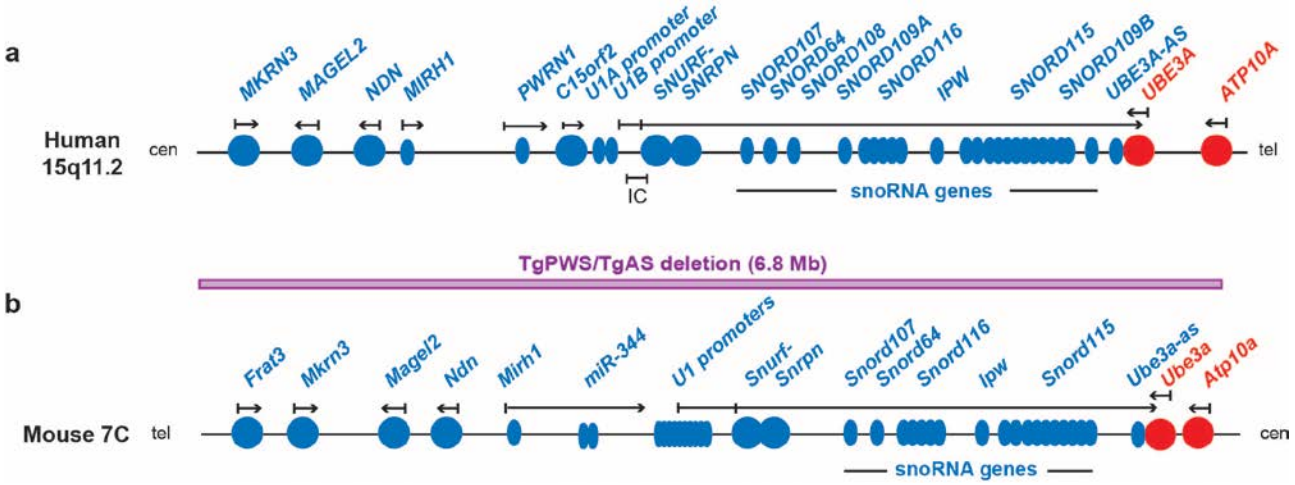


Figure 1.2. Prader-Willi syndrome (PWS) imprinted domain in (a) human chromosome 15q11.2 and (b) mouse chromosome 7C. Genes in blue are paternally expressed, while genes in red are maternally expressed. Circles indicate genes encoding proteins, ovals indicate RNA genes, and arrows indicate the direction of gene transcription. cen, centromere; tel, telomere; IC, imprinting center. Modified from Stefan, *et al*., in preparation.

1.3 CIRCADIAN RHYTHMS

As the word “circadian” was first derived from the latin word “circa diem”, it means “about a day” (Roenneberg & Merrow, 2005; Toh, 2008). Circadian rhythms exist in virtually all living organisms and function as an internal body clock that regulates the metabolic, physical and behavioral paces of the body (Panda *et al*., 2002; Roenneberg & Merrow, 2005; Toh, 2008). The fundamental trigger of circadian rhythms is environmental cues, such as light and temperature (Roenneberg & Merrow, 2005). It is hypothesized that circadian oscillators are an advantage for living organisms to adjust internal physiology to adapt to environmental changes (Panda *et al*., 2002).

1.3.1 Physiology and behavior

The daily sunrise and sunset are considered to be the ultimate force for circadian rhythms (Roenneberg & Merrow, 2005). The central mammalian circadian pacemaker resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (Maywood *et al*., 2007; Takahashi *et al*., 2008). The SCN is composed of bilateral nuclei, each comprising approximately 10,000 neurons which are autonomous cells (Takahashi *et al*., 2008).When these neurons in the SCN are entrained by projection from retinal neurons, they use neuronal and hormonal mechanisms to coordinate rhythmic physiology and behavior. Studies have shown that damage to the SCN eliminates endogenous circadian rhythms, which supports its role as circadian pacemaker (Inouye & Kawamura, 1979). In addition to the SCN, peripheral organs, such as liver and heart, have circadian

rhythms that receive neural and hormonal output signals from the SCN (Rutter *et al*., 2002; Hastings *et al*., 2007) and additionally can also be entrained by food or energy balance (Mendoza, 2007).

In mammals, circadian rhythms influence many aspects of physiology and behavior, including sleep-wake cycles, energy metabolism, hormonal regulation, heart rate, blood pressure and body temperature (Triqueneaux *et al*., 2004; Gallego & Virshup, 2007). Therefore, it is reasonable to consider the goal of circadian rhythms is to optimize metabolism and energy balance for sustaining life of a living organism (Levi & Schibler, 2007).

1.3.2 Molecular clocks in human

In animals, the cellular circadian cycle is determined molecularly by oscillations of positive and negative transcriptional and post-transcriptional feedback regulation. In the positive feedback loop, CLOCK and BMAL1, two basic helix-loop-helix (bHLH)-PAS TFs, form a heterodimeric complex and function as a transcriptional activator in the circadian pacemaker to activate the transcription of circadian core genes, period (PER) and cryptochrome (CRY), as well as other circadian output genes, through E-box DNA binding elements (5'- CACGTG-3', Steeves *et al*., 1999; Gekakis *et al*., 1998; Kondratov *et al*., 2003). On the other hand, PER and CRY also heterodimerize and mediate the negative limb of a transcriptional feedback loop in the circadian system (Sun *et al*., 1997; Darlington *et al*., 1998; Hastings *et al*., 2007; Brown *et al*., 2005). In this feedback system, the CLOCK/BMAL1 complex binds to E-boxes in the promoters of the *PER* and *CRY* genes and stimulates transcription, but as the concentration of PER/CRY increases, they inhibit CLOCK/BMAL1 function. Subsequently, PER and CRY are degraded and cycling of this system generates the circadian oscillations (Reppert & Weaver, 2002). In addition, *NR1D1* (also

known as *REV-ERBα*) forms an ancillary feedback loop by being a repressor of *BMAL1* transcription (Takahashi *et al*., 2008). Another circadian regulator is *DBP*, which encodes a TF that regulates many clock output genes as well as *PER1* (Yamaguchi *et al*., 2000).

Post-translational modifications such as phosphorylation, ubiquitination, sumoylation, and acetylation have been shown to play important roles in the regulation of circadian expression (Cardone, *et al*., 2005; Takahashi *et al*., 2008). Circadian timing of PERs is controlled through progressive phosphorylation by kinases, CSNK1A1 (also known as CKIα, Hirota *et al*., 2010), CSNK1D (also known as CKIδ, Lee *et al*., 2009) and CSNK1E (also known as CKIε, Akashi *et al*., 2002; Takahashi *et al*., 2008; Lee *et al*., 2009). The stability and cellular localization of PER is also regulated by phosphorylation (Akashi *et al*., 2002; Blau, 2008). For CRY1, phosphorylation and protein stability are rhythmically regulated by AMPK, an adenosine monophosphate (AMP)-activated protein kinase (Lamia *et al*, 2009). BTRC (also known as β–TrCP1) and FBXL3, both F-box proteins and subunits of ubiquitin protein ligase complexs, can target phosphorylated PER and CRY proteins, respectively, and promote polyubiquitylation for the 26S proteosomal degradation pathway (Reischl *et al*., 2007; Busino *et al*., 2007). In the circadian system, sumoylation was first found in a lysine residue (Lys^{259}) of BMAL1 by SUMO1 to enhance the circadian expression of BMAL1 (Cardone *et al*., 2005). Such a process is induced by CLOCK (Cardone *et al*., 2005). In addition, CLOCK protein can rhythmically acetylate BMAL1; subsequently, acetylation of BMAL1 facilitates recruitment of CRY1 to CLOCK/BMAL1 for transcription repression (Hirayama *et al*., 2007). SIRT1 (histone deacetylase sirtuin 1), on the other hand, can interact with CLOCK to deacetylate BMAL1 and PER2 (Nakahata *et al*., 2008; Asher *et al*., 2008).

Additionally, histone acetylation and methylation are also crucial in circadian regulation (Takahashi *et al*., 2008; Katada & Sassone-Corsi, 2010). Rhythmic expression of mouse *Per1* and

Per2 are regulated by histone H3 acetylation and deacetylation (Etchegaray *et al*., 2003; Curtis *et al*., 2004; Naruse *et al*., 2004). Similarly, rhythmic histone acetylation was reported in circadian transcription of *Dbp* by the binding of CLOCK/BMAL1 to an intronic *Dbp* enhancer (Ripperger & Schibler, 2006). Later studies showed that CLOCK protein has histone acetyltransferase (HAT) activity (Doi *et al*., 2006; Hirayama *et al*., 2007), while SIRT1 is a NAD+-dependendent deacetylase to histone H3 (Nakahata *et al*., 2008; Asher *et al*., 2008). Circadian expression of SIRT1 was found in mouse liver and fibroblast cells with a constant level of *Sirt1* mRNA, indicating posttranscriptional regulation involved in circadian regulation of SIRT1 (Asher *et al*., 2008). Since the function of SIRT1 depends on NAD+, it provides a link between cellular metabolism and circadian rhythms. Finally, recent studies indicated that a chromatin activator MLL1 is part of the CLOCK/BMAL1 complex and plays a crucial role in circadian transcription and H3K4 trimethylation (Katada & Sassone-Corsi, 2010). Overall, these findings in post-translational regulation and histone modification for chromatin remodeling change the traditional view of the control of molecular clock purely by a transcriptional feedback loop. The network of transcriptional and post-transcriptional regulation shows how complex and hierarchical the molecular control of circadian regulation is. Together, these processes create the dynamic changes of chromatin structure in circadian expression and thus reflected by rhythmic changes on mRNA and protein levels of circadian input and output genes.

During the past decade, studies in circadian rhythms have made remarkable progress. One piece of evidence is that many new genes have been studied and found to play roles in circadian regulation (Asher *et al*., 2008; Lee *et al*., 2009; Katada & Sassone-Corsi, 2010, Hirota *et al*., 2010). However, more evidence indicated that new regulatory genes may involve different levels of regulation to fine-tune circadian regulation (Bozek *et al*., 2010).

1.3.3 Circadian rhythms and disease

Circadian rhythms direct neural and hormonal control of daily activity, feeding, and sleep cycles. They are disrupted by travel, shift-work, and in disease including sleep, eating and psychiatric disorders, obesity, diabetes, cardiovascular disease, infection, and cancer (Kondratov & Antoch, 2007; Weldemichael & Grossberg, 2010; Wulff *et al*., 2010; Gravotta *et al*., 2011).

Since circadian rhythms are highly correlated to body metabolism, many studies showed association between circadian clock and metabolic abnormalities (Bass & Takahashi, 2010; Huang *et al*., 2011). Glucose concentrations in the blood are highly rhythmic (Bass & Takahashi, 2010). Patients with type II diabetes have abnormal circadian insulin secretion (Boden *et al*., 1999). *Clock* and *Bmal1* mutant mice have impaired glucose tolerance, reduced insulin secretion and other phenotypes that lead to the development of diabetes mellitus and obesity (Turek *et al*., 2005; Marcheva *et al*., 2010).

Another common feature for disease associated with circadian rhythms is an abnormality in sleep. The timing of sleep is controlled by the circadian clock with the release of melatonin and a reduction of body temperature. It has been reported that more than 80% of patients with depression or schizophrenia have sleep complaints (Wulff *et al*., 2010). Decrease of melatonin secretion is often found in patients with Alzheimer's disease and phenotypic features of day time agitation, night-time insomnia and restlessness (Weldemichael & Grossberg, 2010; Wulff *et al*., 2010). Similarly, for patients with Parkinson's disease, it is common to have night-time sleep disturbances and daytime sleepiness (Wulff *et al*., 2010).

At the molecular level, the first disorder that linked directly to core circadian genes is familial advanced sleep phase syndrome (FASPS), which has missense mutation in *PER2*

(Takahashi *et al*., 2008). Insomnia is one of the most common sleep disorders and affects approximately 10-15% of the adult population (Ban *et al*., 2011). A cohort study has linked insomnia to SNPs in *ROR1* and *PLCB1*, genes that have previously been associated with bipolar disease and schizophrenia, respectively (Ban *et al*., 2011). A mutation has been found in *DEC2*, encoding a repressor to the CLOCK/BMAL1 complex, in a family-based study of lifelong shorter daily sleep time (He *et al*., 2009). Subjects with the *DEC2* mutation have a short sleep phenotype (He *et al*., 2009).

Interestingly, clinical studies show that disease treatment is often sensitive to the time of administration (Levi & Schibler, 2007). Consequently, rhythmic processes in the body should be considered in personalized pharmacotherapy for more effective treatment (Ohdo, 2010), including in cancer treatment (Kondratov & Antoch, 2007). Therefore, a better understanding of how body clocks work will not only provide insights into the pathogenesis of these and other common disorders, but also improve treatments for disease.

2.0 TRANSCRIPTIONAL AND EVOLUTIONARY MECHANISMS IN PRADER-WILLI SYNDROME

2.1 INTRODUCTION

2.1.1 Regulation of the PWS imprinted domain in somatic cells

As described in **Chapter 1.2.**, PWS is an inborn disease that involves chromosomal abnormalities of human chromosome 15q11.2. In the 2 Mb human PWS domain (**Figure 2.1)**, our laboratory has identified 14 NRF1 binding sites in the *SNURF-SNRPN* promoter (2 sites), *SNURF-SNRPN* intron 1 enhancer (2 sites), a CpG-islet site 3.7 kb 5' of *MKRN3* (intergenic in mouse), the *NDN* promoter, the *U1A* and *U1B* promoters, putative enhancer elements upstream of *U1A* and *U1B*, and in an element termed the "NRF1 cluster". The "NRF1 cluster" element is located between a cluster of intronless genes in proximal 15q11.2 and the *U1-SNURF-SNRPN-snoRNA* region and consists of four conserved NRF1 binding motifs (**Figure 2.2**, Stefan *et al*., in preparation). The stereospecific arrangement of 4 clustered NRF1 motifs suggests binding to the same face of the α-helix and potential for cooperative interactions similar to known enhancer elements (Stefan *et al*., in preparation). Studies by our laboratory also found that the "NRF1 cluster" element shows brain-specific DNA methylation, and has strong enhancer function in reporter assays (Stefan *et al*., in preparation). The "NRF1 cluster" element also shows NRF1 binding and an enhancer-pattern

(Heintzman *et al*., 2007; Schübeler, 2007) of histone H3K4 methylation based on ChIP studies (Stefan *et al*., in preparation). Similarly, in the mouse orthologous region, chromosome 7C, we found 18 NRF1 *cis*-binding sites in 11 gene promoters, two enhancers, and the "NRF1 cluster", all differentially methylated and bound *in vivo* by NRF1 within active chromatin [acetylated histone H4 and dimethylated histone H3 (Lys4)] on the paternal allele only (Stefan *et al*., in preparation). Combined, the human and mouse studies indicated that NRF1 is a "master" regulator of the PWS region (Stefan *et al*., in preparation). However, the previous human studies used SK-N-SH cells which do not express *MAGEL2*, *PWRN1*, or *C15orf2*, and only express a low level of *MIRH1* (Stefan *et al*., in preparation).

In order to confirm and extend analysis of the role of NRF1 in regulating PWS genes, my study has used ChIP assays to examine NRF1 binding in the GM11715 mouse/human somatic hybrid cell line. The GM11715 cell line was derived by microcell fusion of human diploid fetal lung fibroblasts with mouse A9 cells (Ning *et al*., 1992) and is *Hprt*- (Cos *et al.,* 1974), which can be used in drug selection by HAT for gene targeting (Capecchi, 2005). Similarly, A15 (and its derivative, A15-1, which has a truncated version of chromosome 15) is also a somatic cell hybrid derived from mouse A9 cells (McDaniel & Schultz, 1992) but has a more active, hypomethylated epigenetic state in 15q11.2 than does GM11715 (Gabriel *et al*., 1998), which provides alternative cell line(s) for such studies. Expression studies have found that all of the human PWS genes are expressed robustly in the GM11715 cell line with the exception of *MKRN3*; in contrast, the homologous mouse genes are not expressed (**Figure 2.3**). Because the three somatic cell hybrids all express apparently robust levels of PWS genes and contain only one paternally-derived human chromosome 15 (**Figure 2.3**, Gabriel *et al*., 1998), these provide an apparently ideal system for the

study of NRF1 regulation in the PWS domain by gene targeting of a single copy of the "NRF1 cluster".

Gene targeting is based on the principle of homologous recombination, and has been applied to many studies using mouse embryonic stem (ES) cell studies to examine gene function and phenotype, and a few studies in gene regulation (Sedivy & Dutriaux, 1999), as well as studies in somatic cells (Porter & Itzhaki, 1993). If the disruption of the "NRF1 cluster" by gene-targeting was successful, it would allow testing the hypothesis that this element has long-range *cis*-regulatory function in gene expression across the PWS domain. Importantly, this approach will allow analysis of gene expression and changes in chromosomal structure of the entire PWS domain in a chromosome setting.

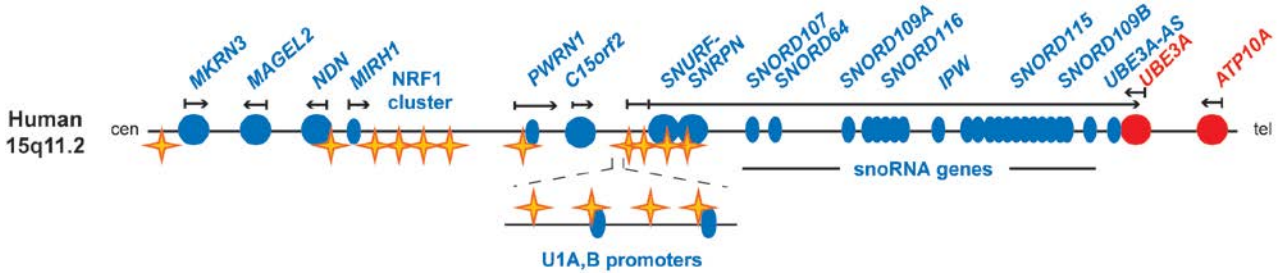


Figure 2.1. NRF1 is a master regulator of the human Prader-Willi syndrome (PWS) imprinted domain in chromosome 15q11.2. Genes in blue are paternally expressed, while genes in red are maternally expressed. Circles indicate genes encoding proteins, ovals indicate genes encoding small RNAs, and arrows indicate the direction of gene transcription. Gold stars indicate verified NRF1 binding sites. cen, centromere; tel, telomere; Modified from Stefan *et al*., in preparation.

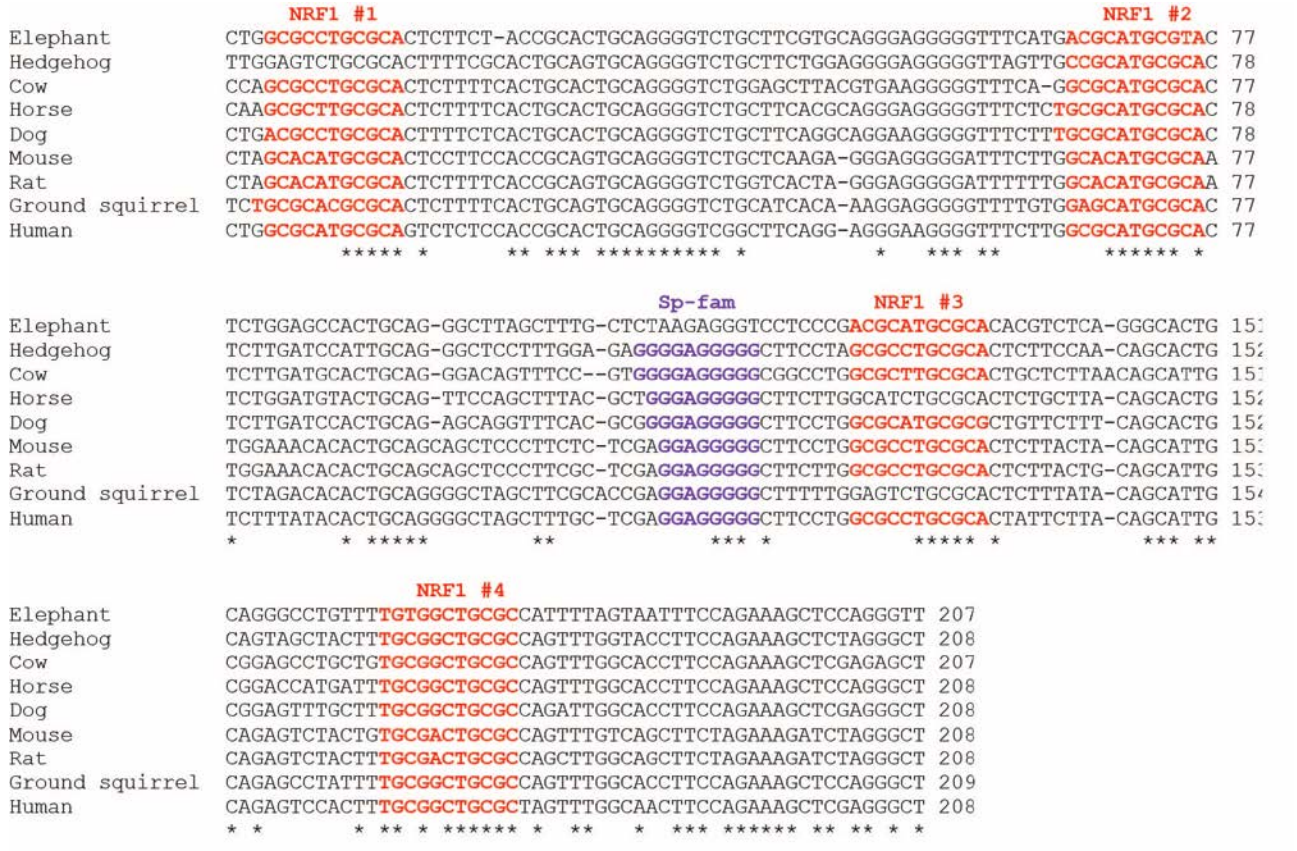


Figure 2.2. Multi-sequence alignment of the "NRF1 cluster" in mammals. NRF1 sites are highlighted in bold red. Sp-family site is highlighted in bold purple.

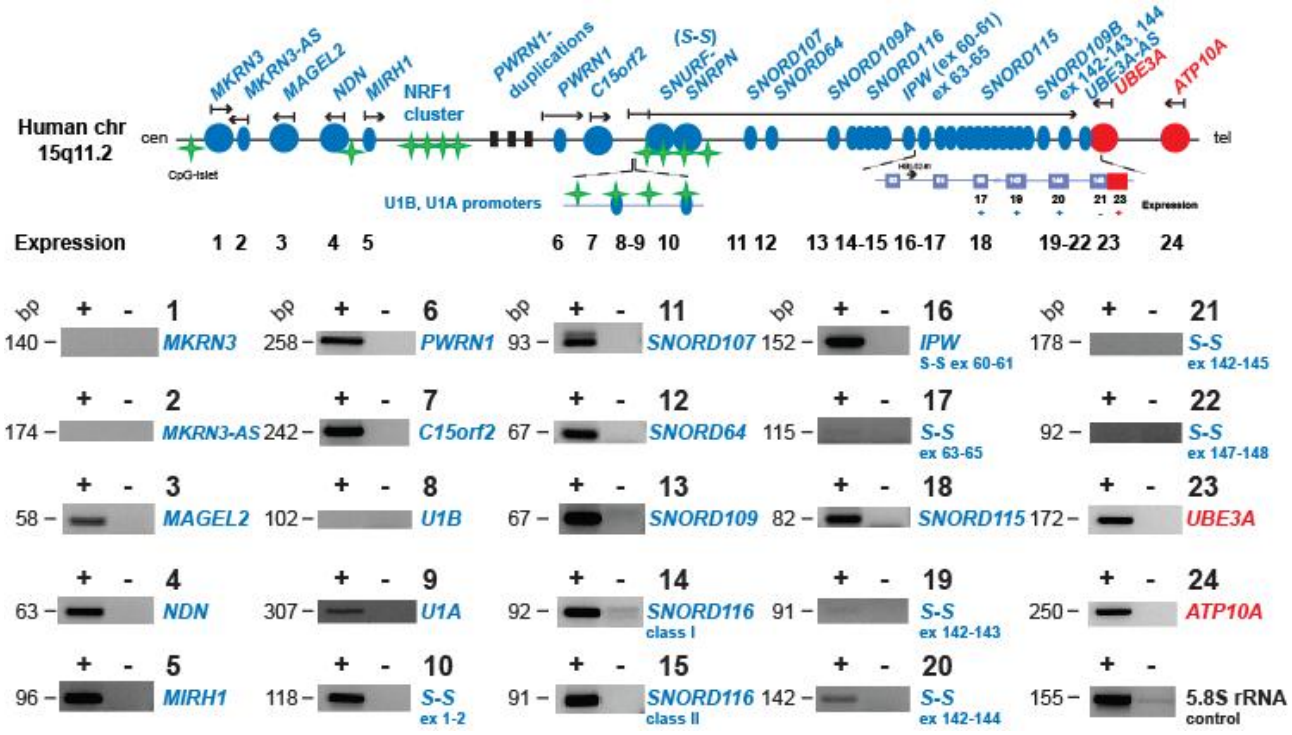


Figure 2.3. Expression studies on GM11715 cells. Abbreviations: "+": RT positive; "-": RT negative; 5.8S rRNA as a positive control (Li, S & R. D. Nicholls).

2.1.2 Genomic imprinting and disease

Genomic imprinting is a type of epigenetic modification that has roles in mammalian development, especially placental and fetal development, childhood and adult behavior (Hall, 1990; Li & Sasaki, 2011). It results in the differential expression of genetic material from the maternally- and paternally-derived alleles. Consequently, genomic imprinting does not show Mendelian inheritance and provides an explanation for some of the genetic events that do not follow single-gene inheritance (Hall, 1990).

Imprinting was first identified during nuclear transfer experiments in mice in the 1980's. By transplantation of either the haploid androgenetic or the gynogenetic nucleus to haploid recipient eggs, only the ones with each copy of paternal and maternal pronuclei resulted in development to term, suggesting the existence of imprinting mechanisms (Surani *et al*., 1986; Ciccone & Chen, 2009). The establishment and removal of genomic imprinting occurs primarily during gametogenesis (but is not completed until early preimplantation development) by epigenetic modification, including DNA methylation by DNA methyltranferases (DNMTs) and DNA demethylases (DMEs), and histone modification by lysine methyltransferases (KMTs) and lysine demethylases (KDMs) (Kota & Feil, 2010; Law & Jacobsen, 2010). Most imprinted genes have regulatory sequences that are differentially methylated regions (DMRs, Uribe-Lewis *et al*., 2011). Once genomic imprinting is established, it is then passed to the zygote through fertilization and maintained throughout somatic development and adult life (Wood & Oakey, 2006; Li & Sasaki, 2011). Only in the formation of primordial germ cells are such modifications erased and new imprints set for the next gametogenesis cycle (Li & Sasaki, 2011). As a result, imprinting maintains permanent genetic features within a generation, but may alter genetic features between generations. The first identified imprinted genes were the paternally expressed insulin-like growth factor 2 (*Igf2*, DeChiara *et al*., 1991) and the maternally expressed insulin-like growth factor type-2 receptor (*Igf2r,* Barlow *et al*., 1991) in mice. To date, 41 transcriptional units (TUs) in human and 71 TUs in mouse have been reported to be imprinted, in which 29 TUs are shared by human and mouse (Morison *et al*., 2001, http://igc.otago.ac.nz/home.html). Among these TU, 30% (25/83) of them encode noncoding RNAs.

Defects in genomic imprinting are associated with different types of disease. Uniparental disomy (UPD) is a condition in which an individual inherited both copies of a chromosome from

one parent only (Yamazawa *et al*., 2010). Usually UPD is not associated with a phenotype unless imprinted genes are present on the chromosome with UPD (or, if UPD results in homozygosity for a recessive mutation). Lack of either paternal or maternal imprinted genes in UPD causes certain inborn diseases. So far, nine types of UPD (Morison *et al*., 2001; Hirasawa & Feil, 2010; Yamazawa *et al*., 2010) have been reported, among which four types are maternal UPD, including Russell-Silver syndrome (or Silver-Russell syndrome, chromosome 7p11-13 and 7q31-qter, Hannula *et al*., 2001; Eggermann, 2010), maternal UPD14 syndrome (MUPD14 syndrome, 14q, Kayashima *et al*, 2002; Ogata *et al*., 2008), PWS (15q11-13, Nicholls *et al*., 1989; Nicholls & Knepper, 2001) and maternal UPD20 (MUPD 20, only three cases, among which one is mosaic trisomy 20 (Chudoba *et al*., 1999; Eggermann *et al*., 2001; Salafsky *et al*., 2001). In addition, five types have paternal UPD, including transient neonatal diabetes mellitus (6q24, Shield, 2000; Aviram *et al*., 2008), Beckwith-Wiedemann syndrome (BWS, 11p15, Smith *et al*., 2007), paternal UPD14 syndrome (14q32, Ogata *et al*., 2008; Yamazawa *et al*., 2010), Angelman syndrome (AS, 15q11-13, Robinson *et al*., 2000; Nicholls & Knepper, 2001) and pseudohypoparathyroidism type 1b (20q13, Morison *et al*., 2001; Amor & Halliday, 2008; Fernández-Rebollo *et al*., 2010). Imprinting abnormalities have also been associated with cancers, which have characteristics of global hypomethylation and site-specific hypermethylation, as well as loss of imprinting in certain types of cancer (Feinberg, 2007). For examples, congenital loss of imprinting at the *IGF2-H19* locus occurs in Wilms tumor and BWS individuals have an increased risk for Wilms tumor and hepatoblastoma (Uribe-Lewis *et al*., 2011). Therefore, a better understanding of the mechanisms of genomic imprinting will not only improve the current functional studies of imprinted genes during gametogenesis and embryonic cell development (Li & Sasaki, 2011), but also improve understanding of mechanisms for some complex diseases.

2.1.3 Evolutionary insights and relationships of the PWS *SNRPN* imprinting locus and snoRNAs

Genomic imprinting is widespread in placental mammals (eutherian mammals) and also occurs in marsupials (metatherian mammals, Renfree *et al*., 2009). These two branches of mammals diverged from a common ancestor about 173-190 million years ago (mya, Kumar & Hedges, 1998; van Rheede *et al*., 2006). The first complete genome sequence of a metatherian, *Monodelphis domestica* (gray short-tailed opossum), revealed that non-protein-coding sequences between metatherians and eutherians were up to 80% conserved (Mikkelsen *et al*., 2007) indicating the high sequence conservation in the gene regulatory elements and their origins from a common ancestor. The study of the metatherian genome provides great insights in understanding the evolutionary history of human genes. As a relatively young genetic mechanism in evolutionary terms, imprinting in mammals has been found to be acquired by a combination of ancient gene functions present in non-mammalian vertebrates and evolutionary young gene loci without clear lower vertebrate ancestors. It has been shown that chicken doesn't have imprinted genes (O'Neill *et al*., 2000). Metatherian imprinted genes include *IGF2* (O'Neill *et al*., 2000; Lawton *et al*., 2008), *PEG1/MEST* (Suzuki *et al*., 2005), *IGF2R* (Killian *et al*., 2001) although it is hypomethylated compared to mouse *Igf2r* (Weidman *et al*., 2006), and *PEG10* (Suzuki *et al*., 2007). The unique and expanded stage of postnatal embryonic development in metatherians provides a good model to study the origin of imprinting from non-placental mammals to placental mammals, since the development of the placenta in eutherians has been suggested to relate to the origin of genomic imprinting (Suzuki *et al*., 2007).

PWS is a genomic imprinting disorder that occurs in approximately 1 per 15,000 newborns. The clinical features of PWS include neonatal failure-to-thrive with childhood-onset severe obesity and other endocrine and behavioral abnormalities. PWS arises from functional loss of multiple imprinted, paternally-expressed genes, which are located on human chromosome 15q11.2 (Nicholls & Knepper, 2001). So far, there are at least thirteen paternally expressed genes that have been identified, including five protein coding genes, *MKRN3*, *MAGEL2*, *NDN*, and a bicistronic *SNURF-SNRPN*. The bicistronic locus *SNURF-SNRPN* normally encodes two polypeptides: *SNRPN* encodes a brain-specific spliceosomal protein, SmN, which replaces SmB'/B protein during postnatal brain development (Sharpe *et al*., 1990; McAllister *et al*., 1989), while the upstream *SNURF* encodes an independent nuclear protein with an unclear function (Gray *et al*., 1999a). *MKRN3*, *MAGEL2* and *NDN* are all intronless genes (Chai *et al*., 2001) and their function remains largely unknown. In mouse, the homologous PWS region is on chromosome 7C, except that mouse doesn't have the testis-specific *C15orf2* (Färber *et al*., 2000), but gained *Frat3* by retrotransposition (Chai *et al*., 2001). The loss of imprinted gene expression on mouse 7C showed similar neonatal defects as in human (Stefan *et al*., 2005). In the PWS gene region, *SNURF-SNRPN* also serves as the host of five classes of C/D-box small nucleolar RNAs (snoRNAs), *SNORD107*, *SNORD64*, *SNORD109*, *SNORD116* and *SNORD115* (Cavaillé *et al*., 2000; Runte *et al*., 2001). SnoRNA is a group of RNA that guides chemical modification of ribosomal RNA (rRNA) or other RNAs. There are two classes of snoRNAs, C/D-box snoRNAs and H/ACA-box snoRNAs. Named by conserved sequence motifs box C/C' (5'-CUGA-3') and D/D' (5'-RUGAUGA-3'), the C/D-box snoRNAs contain one or two 10-21 nucleotide long antisense elements 5' of box C/C', which are complementary to the mature ribosomal RNA and direct site-specific 2'-*O*-methylation (Tyc & Steitz, 1989; Kiss-Laszlo *et al*., 1996; 1998). The C/D-box snoRNAs can be coded either by exonic

sequences (Cavaillé *et al*., 1996) or by intronic sequences (Tycowski *et al*., 1996); however, the latter is more commonly found in vertebrates (Kiss, 2002). For the PWS C/D-box snoRNAs, the function of only *SNORD115* has been identified in the regulation of alternative splicing for the serotonin receptor 2C mRNA (Kishore & Stamn, 2006), and the function of the other four classes of snoRNAs remains unknown. Therefore, understanding the evolutionary origins of PWS genes could provide significant insight for studies of gene function and imprinting mechanism. Previous studies suggested the evolutionary origin of *SNRPN* was ~95-125 mya (Rapkins *et al*., 2006). During evolution, *MKRN3* was derived from *MKRN1* by retrotransposition about 80-120 mya (Gray *et al*., 2000, 2001). *MAGEL2* and *NDN* were derived by retrotransposition from an ancestral X-chromosomal *MAGE* gene (Chai *et al*., 2001; Rapkin *et al*., 2006). The *C15orf2* gene was derived ~40 mya by retroviral insertion and is a primate-specific and intronless gene (Chai *et al*., 2001). Finally, the maternally expressed *UBE3A* and *ATP10C* genes were translocated to the current location (Chai *et al*., 2001). As a model system to understand the acquisition of new gene functions and the origin of genomic imprinting, we have focused on understanding the phylogenetic history of the key gene, *SNRPN*, associated with PWS (Nicholls & Knepper, 2001).

2.2 MATERIALS AND METHODS

2.2.1 Gene expression studies of 3 somatic cell hybrids

RNA was extracted from GM11715, A15, and A15-1 cell lines, and reversed transcribed into cDNA by Super Script®III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The expression of PWS genes in GM11715, A15 and A15-1 cells were tested by primers listed in **Table 2.1** by regular PCR.

2.2.2 Chromatin immunoprecipitation (ChIP) in three somatic cell hybrids

Approximately 1×10^6 GM11715, A15, or A15-1 cells were plated on 35 cm^2 plates with regular media [Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1% penicillin-streptomycin (P/S)]. When cells reached 70-80% confluence, formaldehyde was added to the regular media to make a final concentration of 1% and the plates were incubated at 37°C for 10 minutes to crosslink protein to DNA. After washing with 5 ml ice-cold PBS, another 5 ml ice-cold PBS was added and cells were scraped from the plates using a cell lifter, put into a 15 ml tube and sonicated to shear DNA. Samples were precleared at 4°C with protein G-agarose/salmon sperm beads for 1 h (Millipore). Protein-DNA was immunoprecipitated using the ChIP assay kit (Millipore) with anti-NRF1 (from Dr. Daniel Raines), anti-H3K4me1 [Histone H3 (mono methyl K4) antibody, Abcam, Catalog #: ab8895], anti-H3K4me3 [Anti- trimethyl-Histone H3 (Lys4), Millipore, Catalog #: 07-473], or anti-H3K9me2 [Anti-dimethyl- Histone H3 (Lys9), Millipore, Catalog #: 07-521]. Complexes were collected with Protein G agarose/salmon sperm beads and washed. Protein-DNA complexes were eluted off the beads and cross-links were reversed

by incubation at 65ºC overnight. On the next day, DNA was recovered by phenol-chloroform extraction and precipitated by ethanol. PCR was performed using the recovered ChIP DNA materials as templates and primers from **Table 2.2**. The PCR products were run on an agarose gel to examine the results.

Table 2.1. Primers for gene expression studies using somatic cell hybrids.

Primer name[a]	Primer number	Primer sequence
MKRN3 F	RN2998	5'-CACACGAGAAAGATATGGAAC-3'
MKRN3 R	RN2856	5′-GGAATGGTTGCAATTGGAAAG-3′
MKRN3-AS F	RN2976	5′-TCAAGTCCATCTTTGCTCCAC-3′
MKRN3-AS R	RN2977	5′-CTGTTGAGGCAGTGCTTCTG-3′
MAGEL2 F	RN2853	5′-GTGGAGGCACAGCCCTTGT-3′
MAGEL2 R	RN2854	5′-GGAACTGCACCAACGCATT-3′
NDN F	RN2851	5′-TGCTCCTGCAGAGTTTGGAA-3′
NDN R	RN2852	5′-GGCCTTGACTTTTCTTGGTTAGG-3′
MIRH1 F	RN2921	5′-ATATTGGGGCCTTCGATT-3′
MIRH1 R	RN2922	5′-ACTTGTCGAATTCCATCTGC-3′
PWRN1 F	RN2923	5′-TGTTTCCCAGACCGTAAC-3′
PWRN1 R	RN2924	5′-ACATCCAGTGGTAAGAAT-3′
C15orf2 F	RN2907	5′-CACCAGCAAGCCTATGAATTCC-3′
C15orf2 R	RN2925	5′-GTGGGGATGTGTAGACTGATGTC-3′
U1B F	RN3000	5'-AACTGTGGTCGCTGATCAATG-3'
U1B R	RN2999	5'-TCTGCGTTTGACTTGGACTTCC-3'
U1A F	RN2875	5′-GTCATTCTGCTTGCTGATCAAGA-3′
U1A R	RN2999	5'-TCTGCGTTTGACTTGGACTTCC-3'
SNRPN-SNURF exon 1-2 F	RN3001	5'-TGACGCATCTGTCTGAGGAG-3'
SNRPN-SNURF exon 1-2 R	RN2999	5'-TCTGCGTTTGACTTGGACTTCC-3'
SNORD107 F	RN1455	5'-GGCTAGGTTCATGATGACAC-3'
SNORD107 R	RN1456	5'-GGCACACTGACTGGATTTCA-3'
SNORD64 F	RN1433	5'-GGATTTGTGATGAGCTGTGT-3'
SNORD64 R	RN1434	5'-GGACTTCAGAGTAATCACGT-3'
SNORD109 F	RN1583	5'-GGATCGATGATGAGAATAA-3'
SNORD109 R	RN1584	5'-GAACCTCAGATTGACATGT-3'
SNORD116 class I F	RN1579	5'-ATGATGATGAGTCCCC-3'
SNORD116 class I R	RN1580	5'-GACCTCAGTTCCGATGAG-3'
SNORD116 class II F	RN1581	5'-GATCGATGATGACTTCCA-3'
SNORD116 class II R	RN1582	5'-GGACCTCAGTTCGACGAG-3'
IPW F	RN3002	5'-ATGACTTCCTGGGAACTCTTC-3'
IPW R	RN2874	5′-TGGCACCAACTCAACAAATCC-3′
SNRPN-SNURF exon 63-65 F	RN2970	5′-CTGAAGCTCAGGCCATTCCT-3′
SNRPN-SNURF exon 63-65 R	RN2971	5′-GTCTTCCTCCAGGCTCACTG-3′
SNORD115 F	RN1435	5′-GGGTCRATGATGAGAACCTT-3′
SNORD115 R	RN1436	5′-GGGCCTCAGCGTAATCCTAT-3′
SNRPN-SNURF exon 142 F	RN2972	5′-CACAGCTGACACACCCAGATATC-3′
SNRPN-SNURF exon 143 R	RN2973	5′-CCCTGAAGTTTCCTTGAAGTTGTT-3′
SNRPN-SNURF exon 144 R	RN3069	5′-TGAAGTGGAGGATCAGATTCCAG-3′
SNRPN-SNURF exon 145 R	RN3070	5′-GTTACTTAATCATACAGTAAGCTG-3′

Table 2.1. (continued)

Primer name[a]	Primer number	Primer sequence
SNRPN-SNURF exon 147-148 F	RN2974	5′-AGAAAAGGCGCAATGAAAGA-3′
SNRPN-SNURF exon 147-148 R	RN2975	5′-GGAGAAGGGCCATAGACTCC-3′
UBE3A F	RN2980	5'-AACAAGAAAGGCGCTAGAATTG-3'
UBE3A R	RN2981	5'-TAACTTTCCGGAAGCTCTGTAC-3'
ATP10A F	RN2978	5'-CCTGGCTCAACTGGATAACG-3'
ATP10A R	RN2979	5'-AACTGACGTGCCAGCTGAAG-3'
5.8S rRNA F	RN2849	5'-CGACTCTTAGCGGTGGATCA-3'
5.8S rRNA R	RN2850	5'-GACGCTCAGACAGGCGTAG-3'

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

Table 2.2. Primers for ChIP studies using somatic cell hybrids.

Primer names	Primer number	Primer sequence
SNURF-SNRPN enhancer F	RN2683	5′-CAGGTCTTGGAAGGCTATGTCG-3′
SNURF-SNRPN enhancer R	RN2684	5′-CTCCCCACTGGCGGCTCTAC-3′
SNURF-SNRPN promoter F	RN2877	5′-TGCACTGCGGCAAACAAG-3′
SNURF-SNRPN promoter R	RN3059	5′-AGACAGATGCGTCAGG-3′
U1A promoter F	RN2967	5′-CTGAAGCCTTTGGGATTTTCTA-3′
U1A promoter R	RN2968	5′-AAACAGCTCCAGAGGGAAGAC-3′
U1A (upstream *U1A* promoter) F	RN3266	5′-CGCCATAACCGGAAGAGATC-3′
U1A (upstream *U1A* promoter) R	RN3265	5′-CCCTTTCCCCCTGCTATTTC-3′
U1B (upstream *U1B* promoter) F	RN3263	5′-TGGTCTCCCGTCACCGTTT-3′
U1B (upstream *U1B* promoter) R	RN3264	5′-ACTCCTAGCAGACGCCATAAGC-3′
NDN promoter F	RN2894	5′-CCTAGATCTTCTCAGCCCAAACA-3′
NDN promoter R	RN2895	5′-TCCATGGCGAGGCTTCAC-3′
CpG islet (*MKRN3*) F	RN3033	5′-ATTAAACGCGAGTGTCCAGAAT-3′
CpG islet (*MKRN3*) R	RN3034	5′-CAAACCTGTGAACTAGTCGGTGTA-3′
NRF1 cluster	RN3006	5′-CCTTCTGGCGCATGCGCAGTCTCT-3′
NRF1 cluster	RN2960	5′-AGTGGACTCTGCAATGCTGTAA-3′
MKRN3 promoter F	RN3053	5′-GAAATCGTGTGAGAAGGGACTTAG-3′
MKRN3 promoter R	RN3054	5′-CCTCTGACTGTGTGTTCCTACCTA-3′
MIRH1 promoter F	RN3040	5′-CGTGAGTTAAGAGTGTGAAGGAGA-3′
MIRH1 promoter R	RN3041	5′-CAAACAGTCTGCTCTGAGTCTTGT-3′
PWRN1 promoter F	RN3581	5′-GGCGGTGAGATCTACAGGAG-3′
PWRN1 promoter R	RN3582	5′-GTCTGGGAAACACGGAGAAATC-3′
MAGEL2 promoter F	RN3579	5′-CAGCCTCTGATCCTGCAAATC-3′
MAGEL2 promoter R	RN3580	5′-GTCTGCCAAGTCAGGGGAGTG-3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

2.2.3 Bioinformatics and phylogenetic analyses

Gene sequences were obtained from the Ensembl genome browser and putative NRF1 binding sites were localized within potential gene regulatory regions by examining 5 kb upstream of

the TSS, exon 1, and all introns and 5 kb downstream of 3' UTR of the gene. In this second step, sites matching a GCGCAYGCGC motif, allowing at most one mismatch in one "GCGC" and/or one mismatch in the "AY" segment (on either strand), were selected as potential NRF1 motifs. Second, the sequence spanning the NRF1 motif(s) was used for BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) searches in the non-redundant (NR) and whole genome shotgun (WGS) databases to determine conservation of NRF1 motif(s) across mammalian sequences. Sequences were aligned by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and conserved motifs were highlighted and analyzed by STAMP or other bioinformatic tools.

Genomic sequences for the *M. domestica SNRPN* and *SNRPB'* loci were obtained by NCBI BLAST with the *M. domestica SNRPB'* cDNA sequence (Gray *et al*., 1999b). Sequences spanning each gene were exon-masked and/or Repeatmasked [using the "mammal (other than below)" in "DNA source" option] and used for BLAST. *M. domestica*-specific repetitive sequences were then removed *in silico* when necessary. Candidate non-coding conserved sequences (NCCS) were subsequently analyzed further by BLAST and visual inspection. *SNORD119* snoRNA gene sequences from different species were also obtained from NCBI BLAST searches. *SNORD64*, *SNORD107*, *SNORD109*, *SNORD115* and *SNORD116* human copies were obtained from the NCBI gene database, and other eutherian copies of *SNORD64*, *SNORD107* and *SNORD109* were obtained by BLAST searches in the WGS database. Names of different classes of PWS snoRNAs were used according to official nomenclature from PubMed. *M. domestica SNRPB'* exons and introns sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/index.html). All sequence was transformed into FASTA format using ReadSeq (http://searchlauncher.bcm.tmc.edu/seq-util/readseq.html) and alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The alignment of *SNORD116* was

derived from both ClusterW and Runte *et al*. (2001). Alignments were adjusted as needed to maximize parsimony.

2.2.4 Calculation of minimum free energy in prediction of RNA-RNA interaction

The minimum free energy of interaction for the snoRNA and predicted target RNA was calculated by RNAhybrid (Rehmsmeier *et al*., 2004), which is based on the classical RNA secondary structure prediction algorithm to two sequence (Zuker &Stiegler, 1981) and energy parameters of RNA-RNA interaction by Mathews *et al*. (1999). SnoRNAs and predicted 28S rRNA sequence were submitted in RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) to predict the minimum free energy.

2.2.5 Sequence cloning and analyses

PCR primers (**Table 2.3**) flanking *Didelphis virginiana SNORD119N* and *SNORD119B* were used to clone the respective fragments from *D. virginiana* genomic DNA. The two PCR fragments were then subcloned in to a TOPO TA vector (Invitrogen) for sequencing according to the manufacturer's instructions.

2.2.6 Marsupial, mouse and human RNA and DNA studies

RNA was extracted from *M. domestica* tissue samples with Trizol (Invitrogen) followed by DNase I treatment (Promega). The latter step to remove DNA was critical for studies of small RNA expression where no introns were present. A mouse total RNA panel (10 tissues) and FirstChoice™

Human total RNA survey panel (20 tissues) were purchased from Ambion. PCR and quantitative (Q)RT-PCR primers (**Table 2.3**) were designed by the online primer design program Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and checked by NetPrimer (http://www.premierbiosoft.com/netprimer/index.html). 1 μg of each human, mouse, and *M. domestica* RNA sample was reverse-transcribed (SuperScript II, Invitrogen) into cDNA using hexamer oligonucleotides or specific snoRNA reverse primers in a 20 μl reaction. cDNA was amplified by *Taq* DNA polymerase (Promega), using 94°C for 5 minute, 35 cycles of 94°C for 30 seconds, 50°C (snoRNA primers)/53°C (5.8S rRNA primers)/55°C (*SNRPN*/*SNRPB'* PCR primers) for 30 seconds, and 72°C for 1 minute, then followed by a 10-min final extension at 72 °C. PCR products were analyzed by agarose gel electrophoresis.

2.2.7 Verification of *SNORD119B* and *SNORD119N* snoRNA specific RT-PCR assays by restriction enzyme fragment length variants (RFLV)

The *SNORD119B* and *SNORD119N* snoRNA gene sequences were aligned by Clustal W. The *Hae*III enzyme was chosen to differentiate *SNORD119B* snoRNA from *SNORD119N* snoRNA RT-PCR products. The enzyme digested RT-PCR products were analyzed by agarose gel electrophoresis.

Table 2.3. Primers for evolutionary studies of the PWS SNRPN locus.

Species, target gene, and orientation[a]	Primer	Primer Sequence
Human *SNORD119* snoRNA F	RN2731	5′-GCTGGATTAATGATGAGAT-3′
Mouse *Snord119* snoRNA F	RN2733	5′-GCTGGATTCATGATGAAAT-3′
Human/Mouse *SNORD119* snoRNA R	RN2732	5′-ATCTCAGAGTAATCCTGC-3′
M. domestica SNORD119N snoRNA F	RN2734	5′-GCTGGATTGGTGATGAAACC-3′
M. domestica SNORD119B snoRNA F	RN2736	5′-GCTGGATTAGTGATGAAACA-3′
M. domestica SNORD119N/B' snoRNA R	RN2735	5′-ACCTCAGAGTAATCCTGCT-3′
M. domestica SNRPN (exon 5) F	RN2787	5′-CACAGCCAGTATCGCAGGAGC-3′
M. domestica SNRPN (exon 7) R	RN2788	5′-CAGAGGCAGCATCACAGCAAA-3′
M. domestica s *SNRPB'* (exon 5) F	RN2789	5′-CCCAGGGAAGAGGAACTGT-3′
M. domestica SNRPB' (exon 7) R	RN2790	5′-GCAAGCATCTGAGCAAAGC-3′
Human *SNRPB/B'* (exon 6) F	RN2791	5′-CTGGTATGAGACCTCCTATGG-3′
Human *SNRPB/B'* (exon 7) R	RN2792	5′-CCACAAGGAGATAAAAGGACT-3′
Mouse *Snrpb/b'* (exon 6) F	RN2793	5′-CTGGCCGAGGAACTCCAAT-3′
Mouse *Snrpb/b'* (exon 7) R	RN2794	5′-GGAAGGAAACAGGCAAGGA-3′
Human/Mouse/ *M. domestica* conserved 5.8S rRNA F	RN2849	5′-CGACTCTTAGCGGTGGATCA-3′
Human/Mouse/ *M. domestica* conserved 5.8S rRNA R	RN2850	5′-GACGCTCAGACAGGCGTAG-3′
M. domestica GAPDH cDNA primer (QPCR) F	RN3369	5′-GCCGAGTACGTTGTGGAGTCC-3′
M. domestica GAPDH cDNA primer (QPCR) R	RN3370	5′-AGGGGGCAGAGATAATGACG-3′
M. domestica SNRPN (exon 5 QPCR) F	RN3267	5′-AGCCAGTATCGCAGGAGCC-3′
M. domestica SNRPN (exon 5 QPCR) R	RN3268	5′-GGTGTCCCTCTGCTCACGG-3′
M. domestica SNRPB' (exon 5 QPCR) R	RN3269	5′-AGCCAGCATTGCAGGGGCTC-3′
M. domestica SNRPB' (exon 5 QPCR) R	RN3270	5′-GGTGCCCCTCGACCCATTG-3′
M. domestica SNORD119N (QPCR) F	RN3365	5′-GCTGGATTGGTGATGAAACCTA-3′
M. domestica SNORD119N (QPCR) R	RN3366	5′-CCTCAGAGTAATCCTGCTGAG-3′
M. domestica SNORD119B (QPCR) F	RN3367	5′-GCTGGATTAGTGATGAAACCTG-3′
M. domestica SNORD119B (QPCR) R	RN3368	5′-ACCTCAGAGTAATCCTGCTAAC-3′
D. virginiana *SNRPN* (exon 5) F	RN3362	5′-GCGGTTGCTGCCACAGCCAG-3′
D. virginiana SNRPN (exon 6) R	RN3371	5'-TCCCCATTGGTGCCCCTCGAC-3'
D. virginiana SNRPB' (exon 5) F	RN3364	5′-GCCGCCACAGCCAGCATTGC-3′
D. virginiana SNRPB' (exon 6) R	RN3363	5′-AGGGGGTCTCATGCCAGGAGG-3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

2.2.8 Quantitative analysis of mRNA expression

1 μg of each *M. domestica* RNA sample was reverse transcribed (SuperScript III, Invitrogen) into cDNA using hexamer oligonucleotides and oligo (dT) primers in a 20 μl reaction. Expression of *M. domestica SNRPN*, *SNRPB'*, *SNORD119N*, *SNORD119B* genes using respective primers (**Table 2.3**) was analyzed by quantitative real-time PCR (QPCR) in Applied Biosystems 7300 Real Time PCR system. SYBR green (Power SYBR® Green PCR Master Mix, Applied Biosystems) was used as a fluorescent dye and each cDNA sample was run in triplicate to calculate the statistical significance of the data. Threshold cycle (C_T) was reported from the Applied Biosystems 7300 Real Time PCR system (**Table 2.4** & **2.5**). Outliers in the C_T dataset were determined by Analyze-it for Microsoft Excel 2003 to generate an outlier box plot and the observations outside 1.5 interquartile ranges (IQRs) were defined as outliers. In each tissue sample, the ΔC_T value was obtained by normalizing each gene of interest against *M. domestica GAPDH* data for each assay. The $\Delta\Delta C_T$ value was obtained by subtracting the ΔC_T values for each sample from the ΔC_T values of *GAPDH* in each corresponding assay to calculate a relative $\Delta\Delta C_T$. All $\Delta\Delta C_T$ values were converted to fold change. 2-sample independent t-test was used in Microsoft Excel 2003 to compare the expression difference ($p<0.05$) between 1) *SNRPN* and *SNRPB'*; 2) *SNORD119N* and *SNORD119B* in each tissue sample. Comparisons of gene expression measured by QRT-PCR in brain and non-brain tissues between 1) *SNRPN* and *SNRPB'*; 2) *SNORD119N* and *SNORD119B* were made using 2-way ANOVA (SPSS v13.0) with repeated measures.

Table 2.4. Average C_T for 5 genes in brain tissues of *M. domestica*[a].

C_T	11wk ♀ brain			11wk ♂ brain				Adult ♂ brain			
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
SNRPN	27.64	27.39	28.47	28.83	27.18	28.86	27.15	28.48	27.98	28.90	28.67
SNRPB'	29.00	28.59	29.82	30.23	28.02	29.47	27.88	30.57	29.46	30.72	30.20
SNORD119N	31.67	32.21	33.14	32.39	31.30	33.01	31.75	33.26	32.91	33.07	28.76[b]
SNORD119B	33.66[b]	35.60	35.37	31.62[a]	32.85	33.26	32.00	33.12[a]	34.13	33.24	32.80
GAPDH	22.83	23.19	24.32	24.38	23.72	24.19	22.77	24.40	24.22	25.11	24.42

[a] Abbreviation and symbols: C_T: threshold cycle; wk: week; ♀: female; ♂: male; exp: experiment.

[b] These 3 *SNORD119B* assays were ran separately with *GAPDH* control average C_T values of 22.94, 24.67, 24.75 for 11wk ♀ brain, 11wk ♂ brain, Adult ♂ brain, respectively. [b] The results from this assay were defined as outliers by Analyze-it for Microsoft Excel and not included in the data Figure 2.13.

Table 2.5. Average C_T for 5 genes in non-brain tissues of *M. domestica*[a].

C_T	Liver			Spleen			Kidney			Muscle		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
SNRPN	25.35	24.72	24.84	25.23	25.45	25.86	29.41	28.52	28.71	25.27	27.44	24.88
SNRPB'	24.44	23.68	23.63	23.09	23.08	23.24	28.78	27.53	27.63	25.19	27.28	24.83
SNORD119N	29.95	29.22	29.06	28.38	28.48	28.66	32.00	29.56	31.16	31.93	38.38	33.08
SNORD119B	32.42	31.65	31.62	29.16	29.52	29.43	33.52	33.00	32.54	35.13	38.29	38.93
GAPDH	19.73	19.18	18.92	21.08	21.78	21.76	24.41	23.42	22.96	16.96	20.26	17.06

[a] Abbreviation and symbols: C_T: threshold cycle; exp: experiment.

2.3 RESULTS

2.3.1 Expression profile of PWS genes in three somatic cell hybrids

Expression studies showed that most PWS genes are expressed robustly in GM11715 cells (**Figure 2.3**). In contrast, the homologous mouse genes are not expressed (unpublished data). For A15 cells, previous studies have shown high expression of key PWS genes, including *SNRPN* and *NDN* (Gabriel *et al*., 1998).

2.3.2 NRF1 ChIP results and chromatin status of the 2 Mb PWS region in somatic cell hybrids

Our laboratory has previously demonstrated multiple functional NRF1 sites in the 2 Mb PWS region (Stefan *et al*., in preparation). In this study, I verified the NRF1 regulatory function on PWS genes in a mouse/human somatic cell hybrid cell line, GM11715, by performing NRF1 ChIP assay. As expected, since the *SNURF-SNRPN*-snoRNA polycistronic locus is well expressed in these cells, I found by ChIP assays that NRF1 binds strongly to the *SNURF-SNRPN* promoter and enhancer (**Figure 2.4**). NRF1 also bound to regulatory elements associated with other expressed genes in these cells (**Figure 2.4**), including the *NDN* promoter, the *U1A* promoter and strongly to an upstream putative enhancer, and the *PWRN1* promoter (the first such confirmation), but only weakly to the *MKRN3* enhancer (this Is not surprising since this gene is not expressed in these cells). I also tested the *MAGEL2* promoter, a gene expressed robustly in GM11715 cells, as there

was a weak sequence match to the NRF1 motif but NRF1 does not bind at this element, a result that further helps define functional NRF1-binding motifs. Consistent with no NRF1 binding site in the *MIRH1* promoter, there is no binding of NRF1 (**Figure 2.4**). Surprisingly, since *MIRH1* is expressed at good levels in GM11715 cells, the "NRF1-cluster" (a strong *Mirh1* enhancer *in vitro*) is only very weakly bound by NRF1 despite strong binding of NRF1 to the "NRF1 cluster" in SK-N-SH neuroblastoma cells. Histone code studies have indicated that active chromatin is associated with H3K4me1 (Heinzman *et al*., 2007), and using GM11715 cells I found that all the gene promoters and enhancers in the PWS domain have an active chromatin status [**Figure 2.5(a)**]. Additionally, positive ChIP results using H3K4me3 and negative results with H3K9me2 further support the active chromatin status in this region [**Figure 2.5(b) & (c)]**. Nevertheless, the "NRF1 cluster" and *MKRN3* regulatory regions are only weakly positive. Therefore, we concluded that there are two domains in the 2 Mb PWS imprinted region: one is the *U1A*-*SNURF*-*SNRPN*-snoRNA segment which is highly expressed and strongly binds NRF1 to all promoter and enhancer elements in GM11715 cells, and a more proximal domain containing genes expressed at lower levels (e.g., *NDN*, *MIRH1*) or not at all (e.g., *MKRN3*) with low levels of NRF1 binding (e.g., *NDN* promoter, *MKRN3* enhancer, "NRF1 cluster"). ChIP assays were also performed on both A15 and A15-1 cells to test the binding of NRF1 to NRF1 sites on the human chromosome 15 of PWS region. Unfortunately, our data showed that NRF1 also only bound weakly to the "NRF1 cluster" in the PWS region of A15 or A15-1 [**Figure 2.6(a) & (b)**].

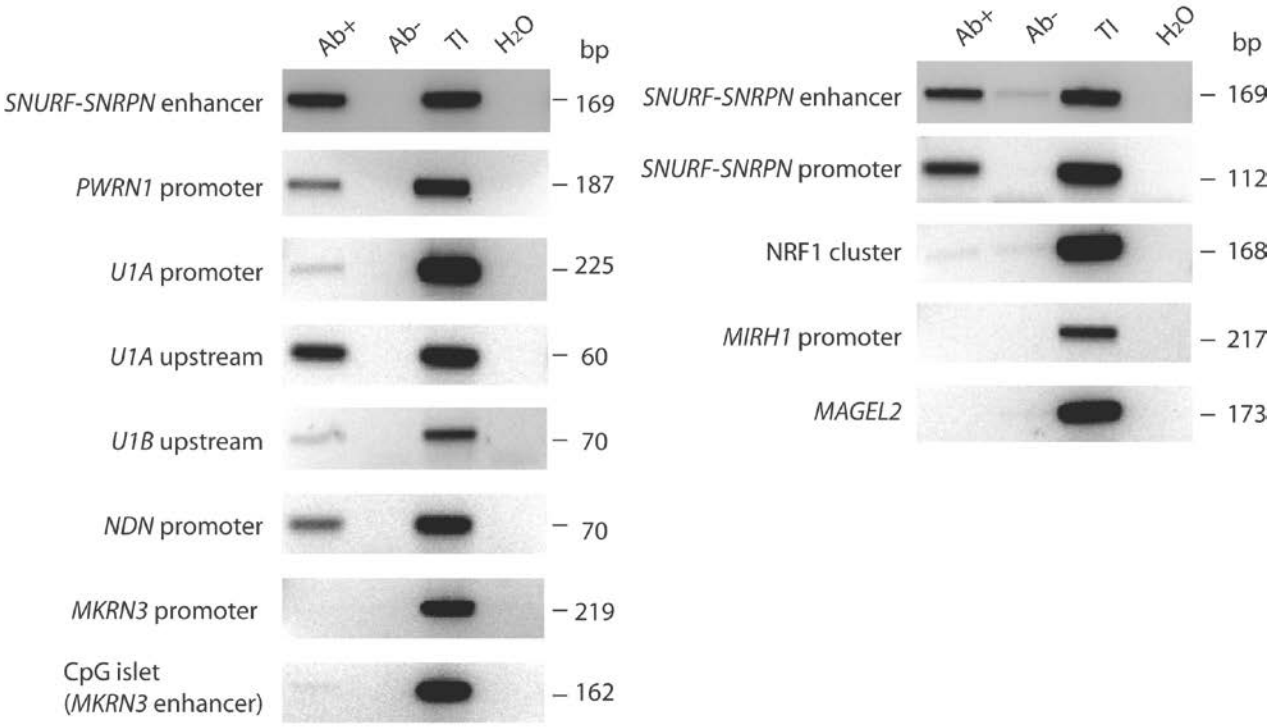


Figure 2.4. NRF1 ChIP assay using GM11715 cells. Abbreviations: Ab+: antibody positive; Ab-: antibody negative; TI: total input; bp: base pair.

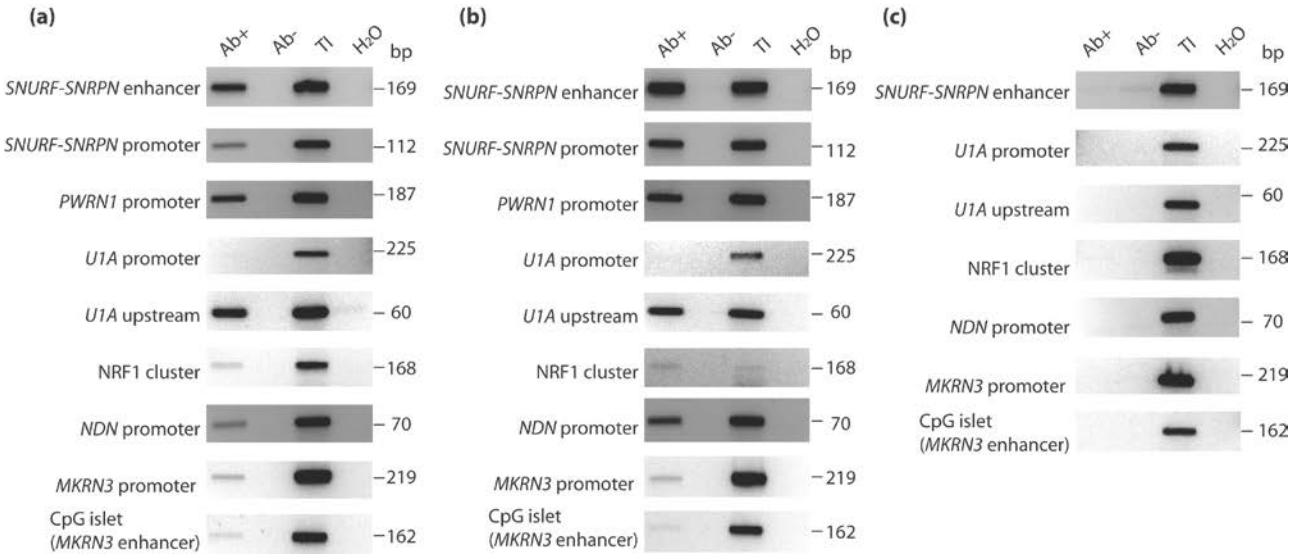


Figure 2.5. ChIP assays using GM11715 cells for (a) H3K4me1, (b) H3K4me3, and (c) H3K9me2 antibodies. Abbreviations: Ab+: antibody positive; Ab-: antibody negative; TI: total input; bp: base pair.

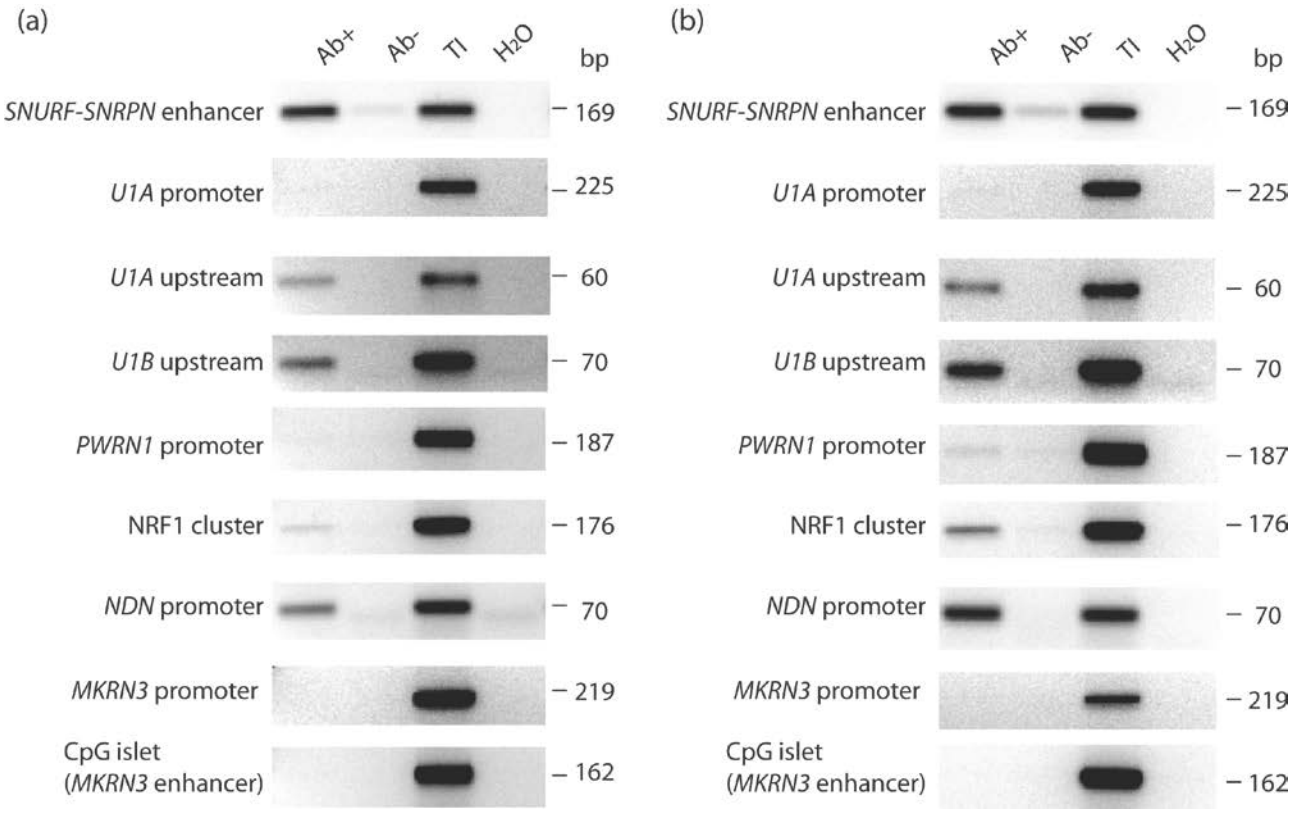


Figure 2.6. NRF1 ChIP assays using (a) A15 and (b) A15-1 cell lines. Abbreviations: Ab+: antibody positive; Ab-: antibody negative; TI: total input; bp: base pair.

2.3.3 Evolutionary studies of the PWS *SNRPN* locus and snoRNAs

2.3.3.1 Intragenic snoRNAs in the duplicated *SNRPB'* and *SNRPN* loci of marsupials

In *M. domestica*, *SNRPN* and *SNRPB'* are adjacent paralogs on chromosome 1 (Rapkins *et al*., 2006). By sequence analysis across species, we found three conserved elements, which were in intron 5 of *SNRPN*, exon 2b of *SNRPB'*, and intron 5 of *SNRPB*' (**Figure 2.7**), respectively. Further analysis confirmed the conserved sequences in intron 5 of *SNRPN* and intron 5 of *SNRPB*' were paralogous genes, both of which were C/D box snoRNAs, and their ortholog in human was named *SNORD119* (Yang *et al*., 2006). Because *SNRPN* and *SNRPB'* were paralogous genes, we named the intron 5 snoRNA in *SNRPN* as *SNORD119N* and the intron 5 snoRNA in *SNRPB'* as *SNORD119B*. To further examine these results in other marsupial species, we verified the existence of the snoRNAs in *Didelphis virginiana* (Virginia opossum) by PCR cloning and in *Macropus eugenii* (tammar wallaby) by sequence BLAST (**Figure 2.8**).

2.3.3.2 Molecular evolution of the *SNORD119* snoRNA within *SNRPB'* intron 5 in vertebrates

Since the protein of *SNRPB'*, SmB', is an indispensable protein that constitutes one of the major proteins in the assembly of the Sm spliceosome (Matera *et al*., 2007) and snoRNAs have important functions in rRNA modification (Maden, 2001), it was not surprising that we found *SNRPB'* conserved across species, from yeast to eutherian mammals. *SNORD119B* orthologs were found consistently in intron 5 of the *SNRPB'* gene of all analyzed eutherian mammals, reptiles, *Xenopus tropicalis*, and zebrafish, in intron 6 of the *SNRPB'* gene of Atlantic salmon, but not in any intron of birds [**Figure 2.9(a)**].

By sequence BLAST, we determined that genes (*CD20-ELMO2*) upstream of *M. domestica SNRPN* were syntenic to human chromosome 20q13.12 and to mouse 2H3, while those downstream

of *M. domestica SNRPN* (*SNRPB'-STK35*) were syntenic to human chromosome 20p13 and to mouse 2F3, and the block of *M. domestica SNRPN* was syntenic to the PWS region of human chromosome 15q11.2 and to mouse 7C (**Table 2.6**). We found large blocks of repetitive elements on the 5' upstream (~47k, 96% repetitive) and 3' downstream (~6k, 67% repetitive) sides of *M. domestica SNRPN*, respectively (**Table 2.6**).

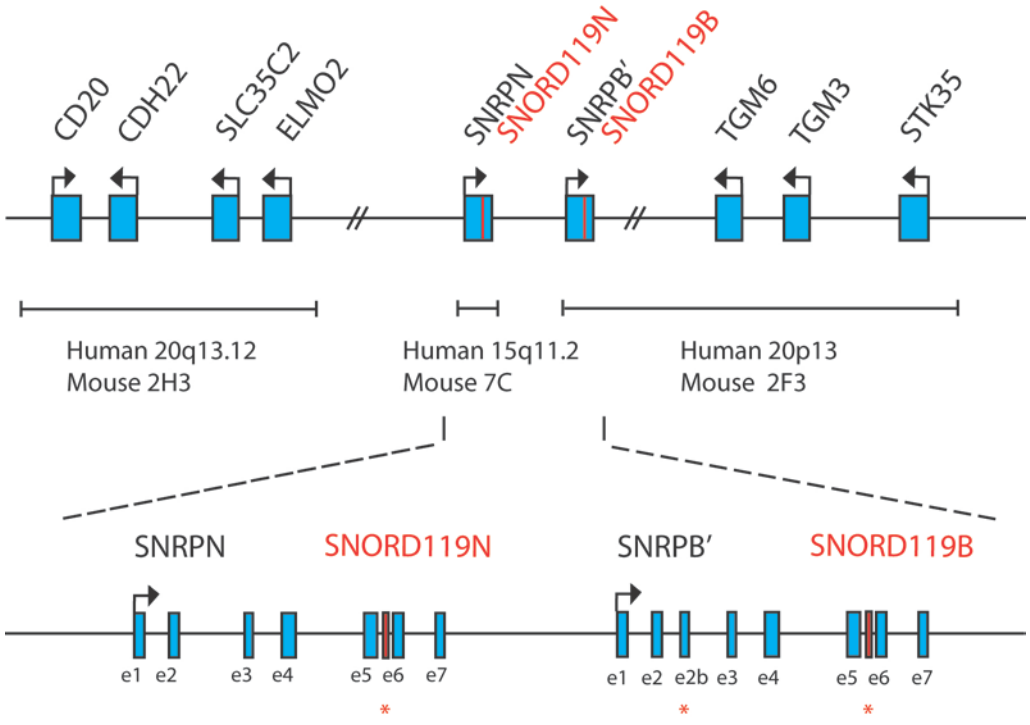


Figure 2.7. Genetic maps of the *SNRPN--SNORD119N* and *SNRPB'--SNORD119B* loci, and syntenic sequences in human and mouse. Arrows indicated transcriptional direction. Red asterisks indicated conserved regions.

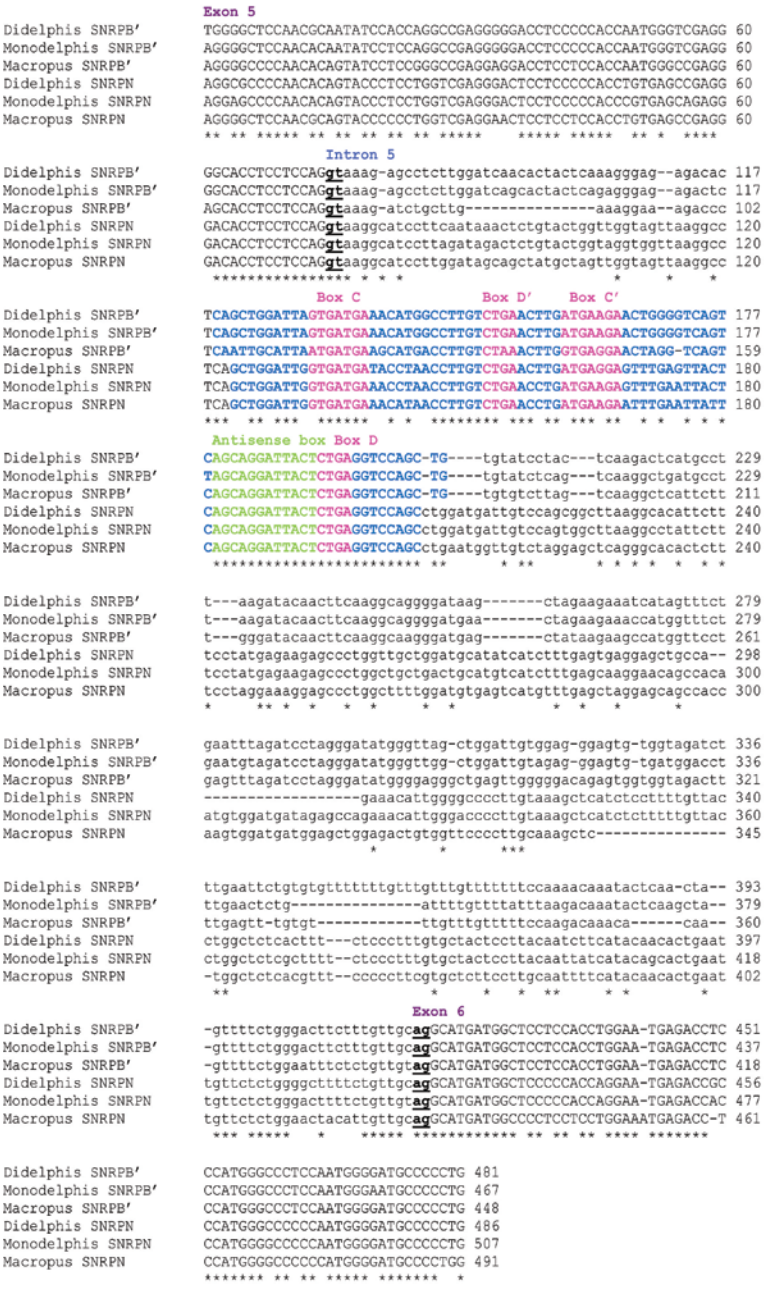


Figure 2.8. DNA sequence comparison of *SNORD119B* and *SNORD119N* in three species of metatherians, *M. domestica*, *D. virginiana*, and *M. eugenii*. Intron sequence is in small case, with exon-intron and intron-exon boundaries were in bold black and underlined. The *SNORD119N* and *SNORD119B* alignment is in bold blue. Box C/C' and box D/D' are in bold pink and the antisense box is in bold green.

Table 2.6. Analysis of the *ELMO2-SNRPN-SNRPB'-TGM6* genomic interval in *M. domestica*.

Region	Extracted feature[a]	Size (bp)[a]	Repetitive	Unique	Features identified
ELMO2-SNRPN	None	47,876	96%	4%	Gene promoters & FLJ13305 pseudogene[b]
SNRPN intragenic	Exons, snoRNA	12,976	44%	56%	*TAF9* pseudogene[c]
SNRPN-SNRPB'	None	6,210	67%	33%	None
SNRPB' intragenic	Exons, snoRNA	10,530	36%	64%	None
3' of *SNRPB'*	None	28,470	69%	31%	3' end of *TGM6* (exon 9-12)

[a] Size of region analyzed after extracting (masking) exons and snoRNAs. For this analysis, we masked *SNRPN* exons 1-7, *SNRPB'* exons 1-7, *SNRPB'* exon 2b, *SNORD119N* and *SNORD119B* sequences.

[b] The *SNRPN* and *ELMO2* promoters have only 363-bp and 659-bp unique sequence, respectively. The *ELMO2* promoter has two elements conserved in mammals. The 1.55 kb FLJ13305 pseudogene is located 36.2-kb 5' of *SNRPN*.

[c] Located in *SNRPN* intron 4, on the complementary strand.

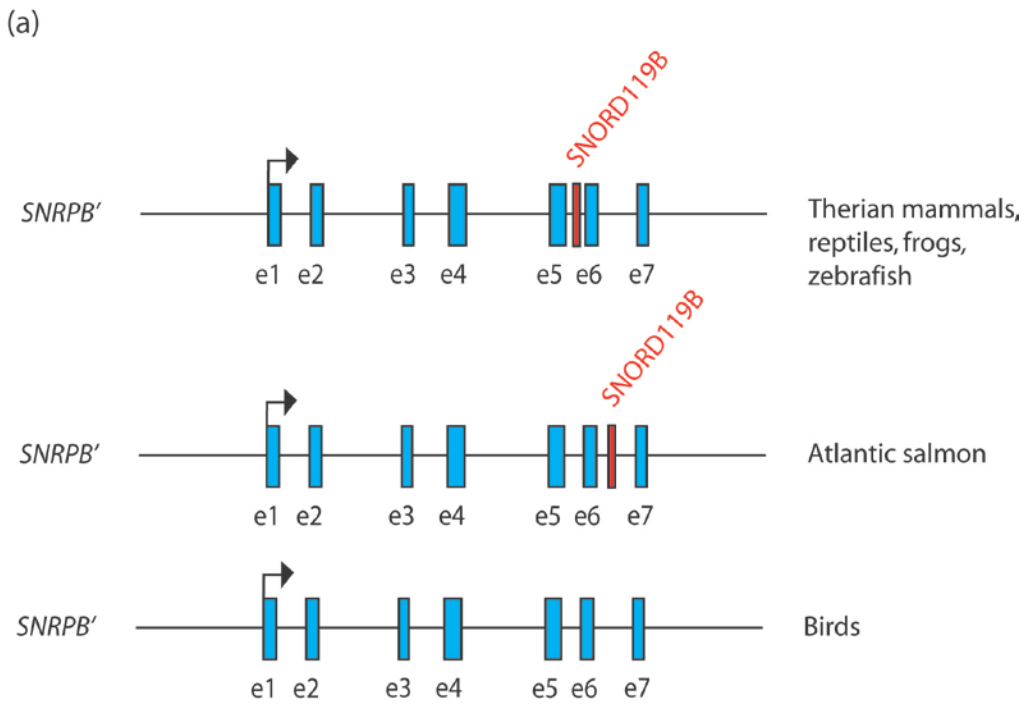


Figure 2.9. (continued below)

(b)

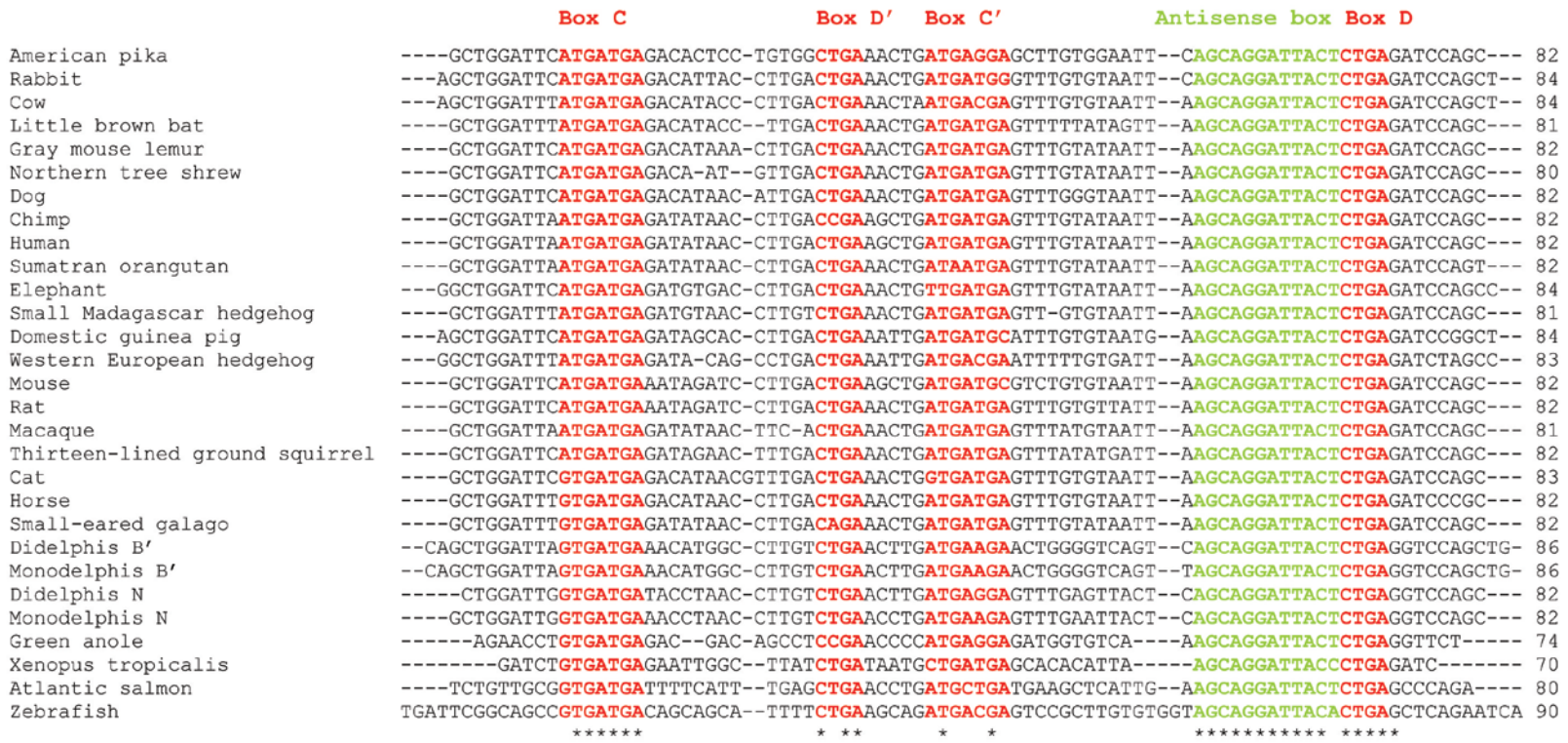


Figure 2.9. Phylogenetic analyses of the *SNORD119B* snoRNA. (a) Cartoon of *SNRPB'* gene structure of therian mammals, reptiles, an amphibian and zebrafish with the *SNORD119* snoRNA in intron 5, whereas the *SNORD119* snoRNA is in intron 6 of Atlantic salmon. In contrast, the *SNRPB'* gene in birds has no intragenic snoRNA. (b) Clustal W multisequence alignment of *SNORD119* snoRNAs. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

2.3.3.1 Expression studies of *SNORD119* orthologs and paralogs

Expression studies by RT-PCR showed that *SNORD119* snoRNA was ubiquitously expressed in 20 analyzed human tissues [**Figure 2.10 (a)**] and 10 analyzed mouse tissues [**Figure 2.10(b)**]. In *M. domestica*, *SNORD119N* and *SNORD119B* were ubiquitously expressed in 8 tissues from one female animal and 4 brain tissue samples from 4 animals (11 weeks postpartum male and female, adult male and female, respectively) [**Figure 2.11(a) & (b)**]. Because *SNORD119N* and *SNORD119B* were highly conserved sequences (**Figure 2.8**) and the PCR primers for *SNORD119N* and *SNORD119B* only differ in two non-contiguous nucleotides, to confirm we had tested specific gene expression, we used *Hae*III enzyme digestion on both PCR products and verified the primers were specific to *SNORD119N* and *SNORD119B* PCR products [**Figure 2.12(b)**] in *M. domestica*.

By quantitative real-time PCR (QRT-PCR) studies of gene expression in *M. domestica*, our results showed that in 3 brain tissues, *SNRPN* was expressed at a higher level than *SNRPB'* (11 weeks postpartum female, $P<0.05$, and adult male, $P<0.001$, but not in 11 weeks postpartum male, $P=0.116$), while in the 3 non-brain tissues, liver, kidney and spleen, *SNRPB'* expression was significantly higher than *SNRPN* [liver, spleen, and kidney, all $P<0.05$; **Figure 2.13(a)**]. These results were consistent with protein studies of SmN and SmB in mice (Gray *et al*., 1999b). In addition, in 3 of 4 female tissues (one juvenile brain and all non-brain tissues were from the same adult animal) analyzed, *SNORD119N* gene expression was significantly higher than *SNORD119B* (11 weeks postpartum female, $P<0.001$; liver, $P<0.0001$; spleen, $P<0.05$; but in kidney, $P=0.141$), except in two male brain samples [11 weeks postpartum male, $P=0.990$, and adult male, $P=0.808$, **Figure 2.13(b)**]. This suggests a different expression level of *SNORD119N* and *SNORD119B* in male versus female in *M. domestica*; however, larger numbers of animals would be needed to

further examine this possibility. By 2-way ANOVA analyses to compare gene expression in brain tissues, *SNRPN* gene expression was significantly higher than *SNRPB'* [F(2, 1)=31.10, $P<0.0001$), but *SNORD119N* and *SNORD119B* gene expression was not [F(2, 1)=0.71, $P=0.414$) due to the lack of expression difference in the two male brain tissues [**Figure 2.13(b)**]. Similarly, 2-way ANOVA analyses comparing gene expression in non-brain tissues (liver, spleen and kidney) showed that *SNRPN* expressed significantly lower than *SNRPB* [F(2, 1)=45.08, $P<0.0001$] and *SNORD119N* expressed significantly lower than *SNORD119B* [F(2, 1)=9.08, $P=0.011$].

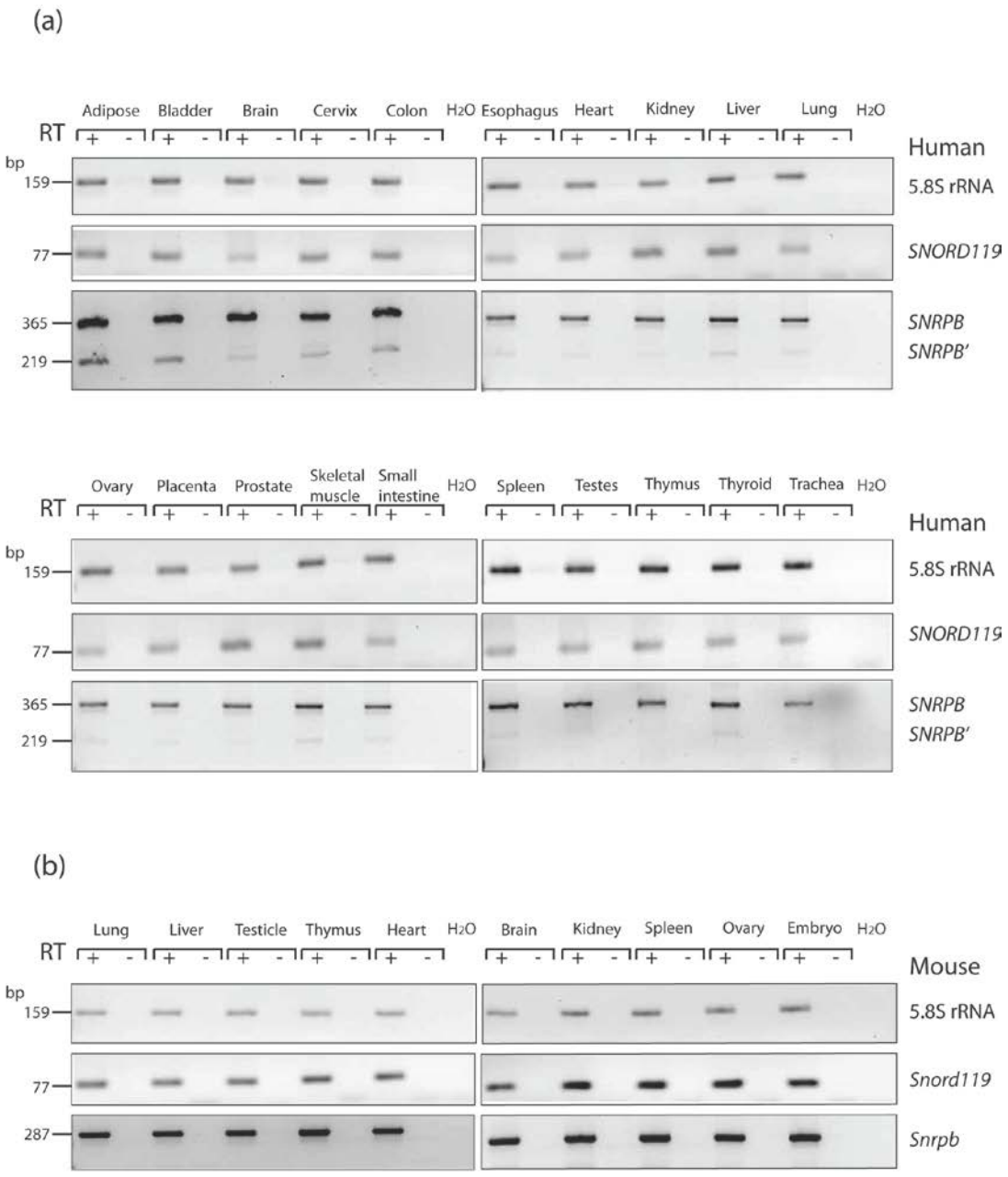


Figure 2.10. Expression studies of *SNORD119*, *SNRPN* and *SNPRB'* in human and mouse. (a) RT-PCR in 20 tissues from human. **(b)** RT-PCR in 10 tissues of mouse. Abbreviations: RT +: reverse transcription positive sample; RT -: reverse transcription negative control; bp: base pair.

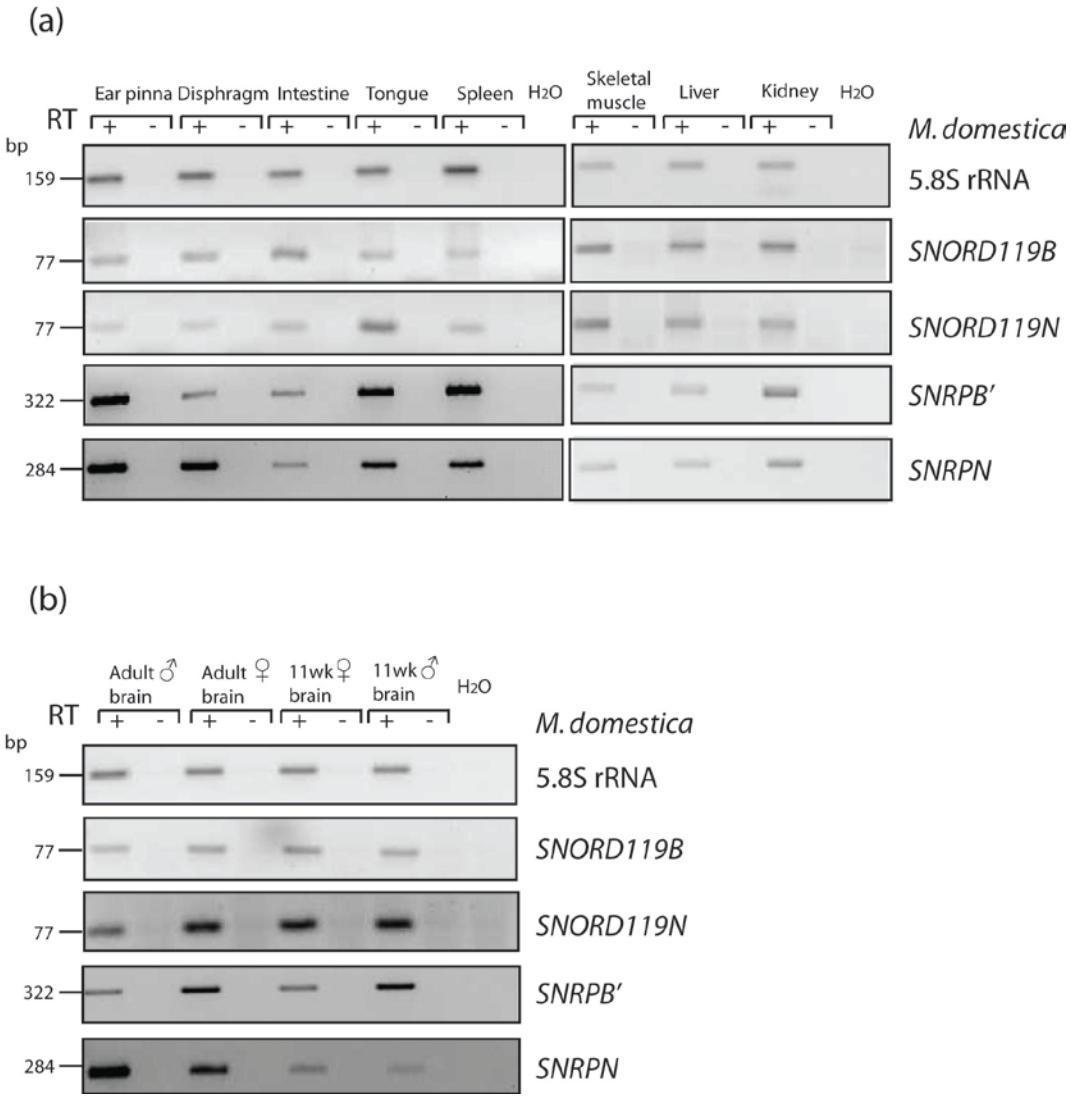


Figure 2.11. Expression studies of *SNORD119N*, *SNORD119B* and their host genes in *M. domestica*. (a) RT-PCR in 8 non-brain tissues of *M. domestica*. **(b)** RT-PCR in 4 brain tissues of *M. domestica*. Adult 1 ♂: animal #J7614, male; Adult 2 ♀: animal #J7344, female; 11 wk ♀: juvenile *M. domestica,* 11 weeks postpartum, female; 11 wk ♂: juvenile *M. domestica,* 11 weeks postpartum, male.

Figure 2.12. Verification of *SNORD119B* and *SNORD119N* snoRNA-specific RT-PCR assays by restriction enzyme fragment length variants (RFLV). (a) Alignment of the snoRNA sequences, with the position of RT-PCR primers in blue rectangles and the recognition site for the *Hae*III restriction enzyme in a red rectangle. (b) RFLV analysis on PCR products from RT-PCR using *SNORD119B*-specific and *SNORD119N*-specific primers. Abbreviations: Ta: annealing temperature in PCR; *Hae*III +: PCR product was digested by *Hae*III; *Hae*III -: PCR product was not digested, as a negative control.

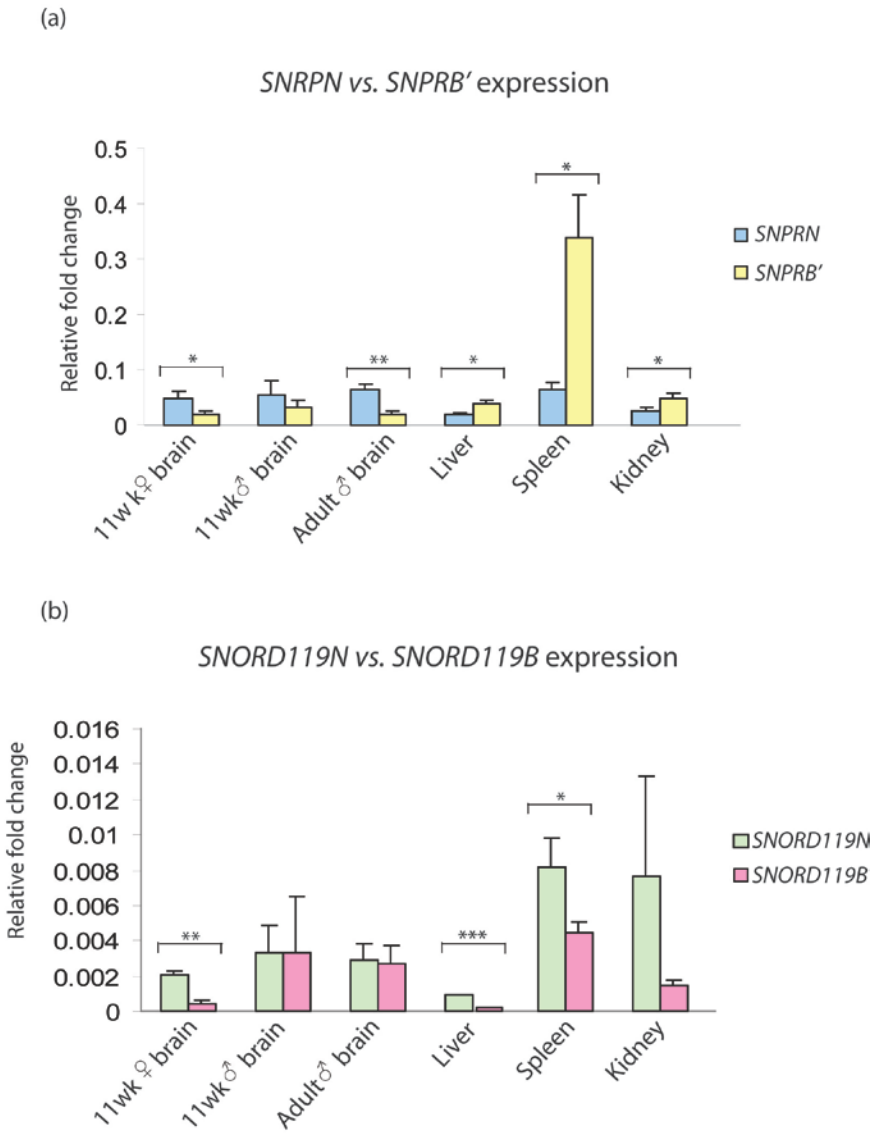


Figure 2.13. Expression studies of *SNORD119* snoRNAs by QRT-PCR in *M. domestica*. Abbreviations: Adult ♂: animal #J7614, male; 11 wk ♀: juvenile *M. domestica,* 11 weeks postpartum, female; 11 wk ♂: juvenile *M. domestica,* 11 weeks postpartum, male. Liver, spleen and kidney were from a female adult animal #G3232. Group comparisons of (a) *SNRPN* and *SNRPB'*; and (b) *SNORD119N* and *SNORD119B* were calculated by 2-sample t-tests using Microsoft Excel 2003. *P* values <0.05, <0.001, <0.0001 are represented by "*", "**" and "***", respectively.

2.3.3.2 Function of *SNORD119* orthologs and paralogs

As noted above, the C/D box snoRNAs have one or two 10-21 nucleotide long antisense elements 5' of box C/C', which are complementary to the mature rRNA and guide site-specific 2'-*O*-methylation. Structural conservation of the core of rRNA and modification is conserved between *Xenopus laevis* and human (Maden, 1990). In the same study, by oligonucleotide fingerprinting, the 4560 position of 28S rRNA was methylated in both human and *Xenopus laevis*; however, the same position was unmethylated in chicken 28S rRNA (Khan & Maden, 1976; Maden & Hughes, 1997, listed as position 4550 in their paper). When we aligned *SNORD119B* antisense box sequence to rRNA position 4560, we found the interaction was thermodynamically favorable (**Figure 2.14**). Similarly, *SNORD119N* antisense box was also thermodynamically favorable in aligning to the same position of rRNA in *M. domestica*. Therefore, we predicted the function of *SNORD119B* and *SNORD119N* is to target rRNA 4560 site for 2'-*O*-methylation (**Figure 2.14**). However, experimental studies are needed to test which snoRNA targets 28S rRNA *in vivo*. It is likely that *SNORD119B* is the common form and functions in most circumstances, while *SNORD119N* may function in the brain.

Similar targeting sequence was found in human 28S by *SNORD119* (**Figure 2.15**). Such interaction was thermodynamically favorable. Surprisingly, we found that SNORD115-3 was also thermodynamically favorable in potentially targeting the same position of 28S rRNA (**Figure 2.15**).

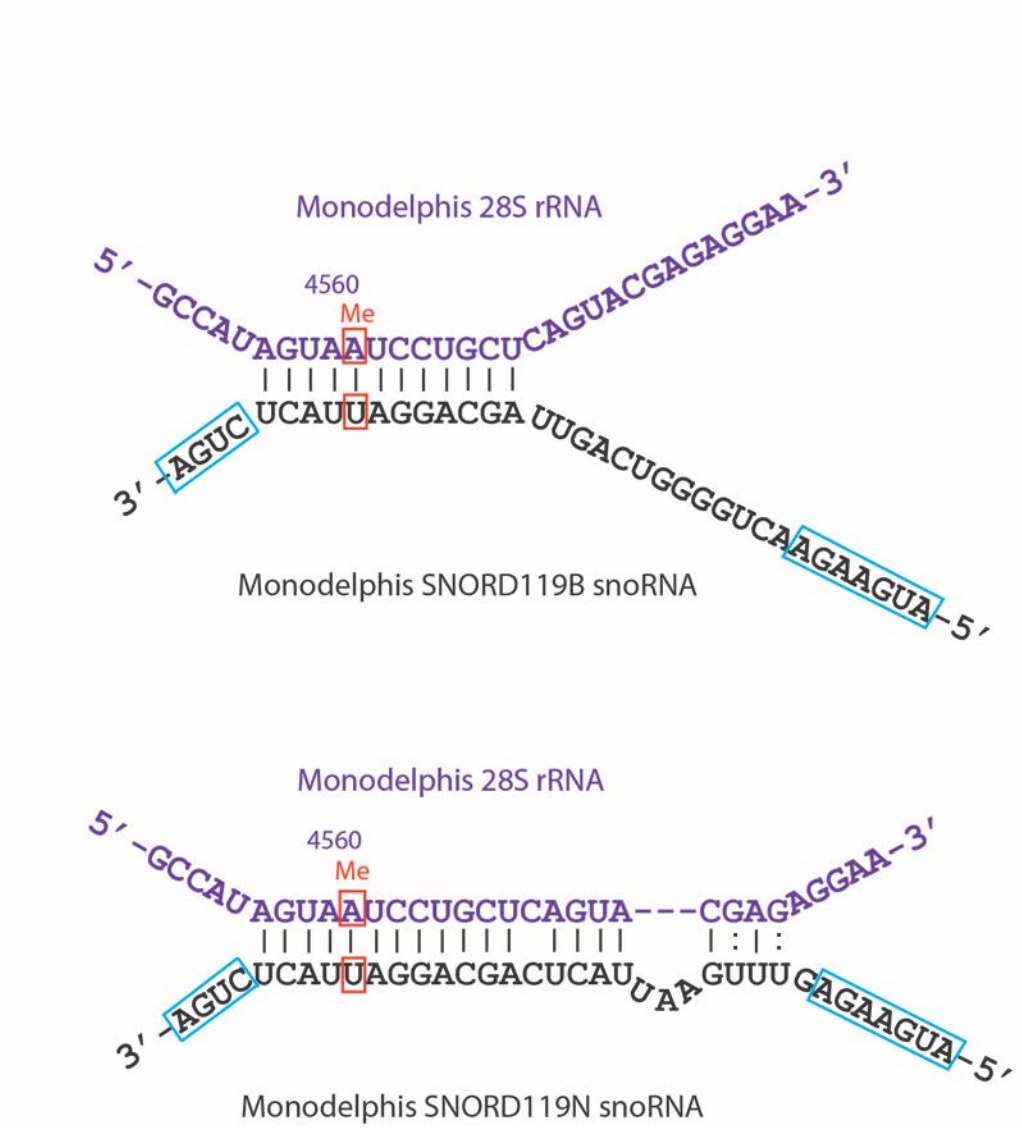


Figure 2.14. Predicted *M. domestica* 28S rRNA targeting by *SNORD119B* and *SNORD119N*. Box C' and box D are shown in blue rectangles. The predicted minimum free energy of *SNORD119B* binding to *M. domestica* 28S rRNA is ΔG°(37°C)=-23.6 kcal/mol, and for *SNORD119N* binding to *M. domestica* 28S rRNA is ΔG°(37°C)=-28.3 kcal/mol.

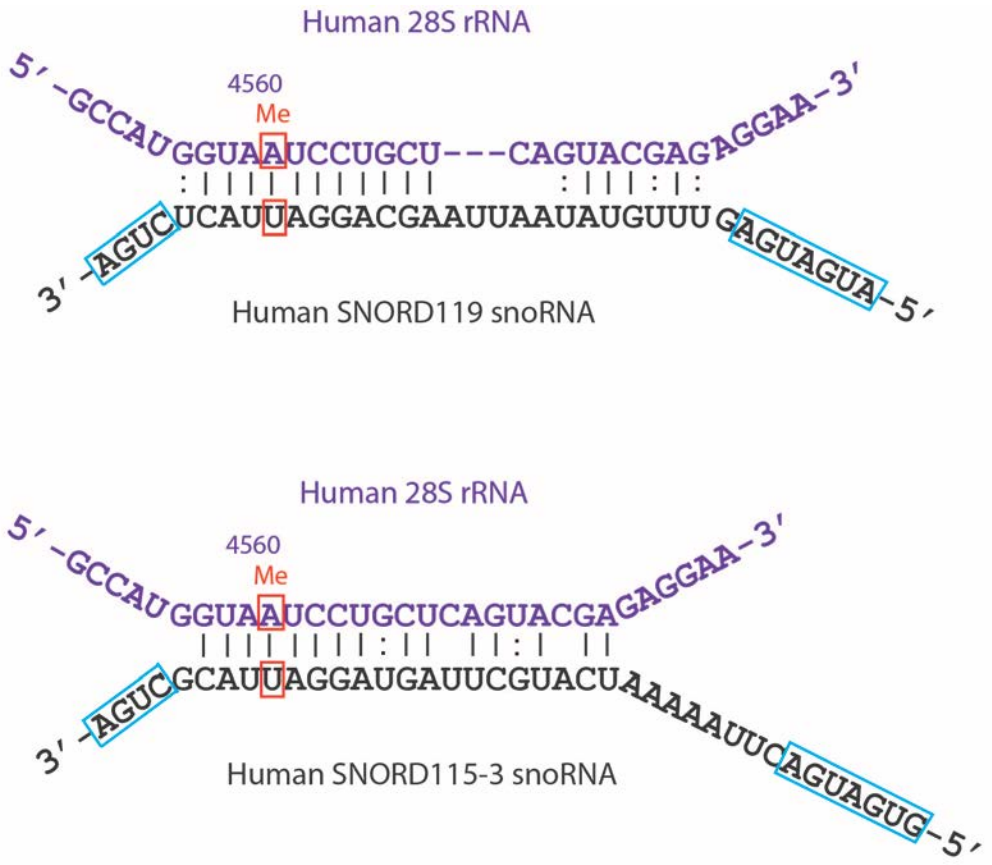


Figure 2.15. Comparison of human *SNORD119* and *SNORD115-3* for their potential targeting of 28S rRNA. Box C' and box D are shown in blue rectangles. The predicted minimum free energy of *SNORD119* binding to 28S rRNA is ΔG°(37°C)=-28 kcal/mol, and for *SNORD115-3* binding to 28S rRNA is ΔG°(37°C)=-26.9 kcal/mol.

2.3.3.3 Molecular phylogeny of *SNORD119* family descendants: the PWS imprinted snoRNAs

Unlike *M. domestica,* in human and mouse, *SNRPN* is located on a different chromosome to *SNRPB'* and forms a more complicated bicistronic locus, *SNURF-SNRPN,* in the PWS domain. The adjacent gene structure of paralogous *SNRPN* and *SNPRB'* only exists in metherians (both *M. domestica* and *D. virginiana*), but not in birds, reptiles, and platypus (data not shown), indicating the origin of the *SNRPN* occurred in a common ancestor of therians.

Downstream of the *SNURF-SNRPN* bicistronic locus in the PWS region, there are five classes of C/D-box snoRNAs in the intronic regions of extended host transcripts derived from alternative *SNURF-SNRPN* promoters (**Figure 2.16**). Among the five classes of PWS C/D-box snoRNAs, there are some copy number variances in different classes. *SNORD64* and *SNORD107* each has one single copy. *SNORD109* has two identical copies A and B. *SNORD115* has 47 copies. *SNORD116* has 29 copies (**Figure 2.16**, Cavaillé *et al*., 2000; Runte *et al*., 2001). In our analysis, we found that *SNORD64* was highly similar to *SNORD115*, *SNORD107* was highly similar to *SNORD109*, while *SNORD115* was also related to *SNORD109* or *SNORD107* (**Figure 2.17**). The three sub-classes of *SNORD116* are significantly more divergent to the other groups of PWS C/D-box snoRNAs. To better assess the sequence comparison and evolutionary studies between *SNORD119N* and PWS C/D-box snoRNAs, we BLASTed human *SNORD64*, *SNORD107* and *SNORD109* in the WGS database in NCBI and aligned maximum matched sequences from difference eutherian species to the human sequences, then achieved eutherian consensus sequences for *SNORD64*, *SNORD107*, and *SNORD109* (**Figure 2.18**). *SNORD119N* snoRNA showed identity to each class of PWS C/D-box snoRNAs (**Figure 2.19**). Combined, the information on the similarity between *SNRPN* versus the *SNURF-SNRPN* bicistronic locus and *SNORD119N* snoRNA versus the five classes of PWS C/D-box snoRNAs, suggested that the eutherian *SNURF-SNRPN*-

snoRNA locus evolved from a common ancestor as did the metatherian *SNRPN-SNORD119N* locus.

Based on these analyses, a gene evolutionary model (**Figure 2.20**) was established. In this model, we predicted that about 173-190 mya, the common ancestor of therians had only one copy of *SNRPB'*. The ancestral copy of *SNRPB'* was duplicated to form a paralogous gene, *SNRPN*, upstream of the ancestral *SNRPB'*. *SNRPN* then became fixed evolutionarily due to gain of functional requirement and selective pressure. In metatherians, the adjacent paralogs of *SNRPN* and *SNRPB'* remains in the same position. In a eutherian ancestor, however, *SNRPN* was translocated to another chromosome, and ontained *SNURF* by *de novo* gene formation or an unknown mechanism (Gray *et al*., 1999a; Chai *et al*., 2001). During the evolution from eutherian ancestor to modern eutherians, other chromosomal rearrangement occurred. In our model, combined with other evolutionary studies, *MKRN3* was acquired by retrotransposition from an ancestral *MKRN1* gene (Gray *et al*., 2000, 2001), and the intronless *NDN* and *MAGEL2* were acquired by retrotransposition from an ancestral *MAGE* gene on the X chromosome and subsequent duplication (Chai *et al*., 2001; Rapkins *et al*., 2006). Subsequent to multiple duplication and diversification events, the *SNORD119* gene gave rise to the five classes of PWS C/D-box snoRNAs, although these have unknown functions. Additionally, the *SNORD115* and *SNORD116* C/D-box snoRNA families have duplicated many times to form different sub-classes (**Figure 2.21 & Figure 2.2).**

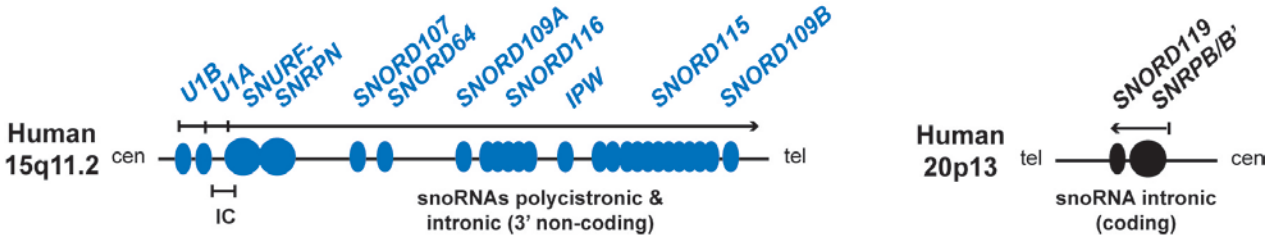


Figure 2.16. Map of PWS C/D-box snoRNAs. Circles represent protein coding genes, and ovals represent RNA genes. Abbreviations: IC: imprinting center; cen: centromere; tel: telomere.

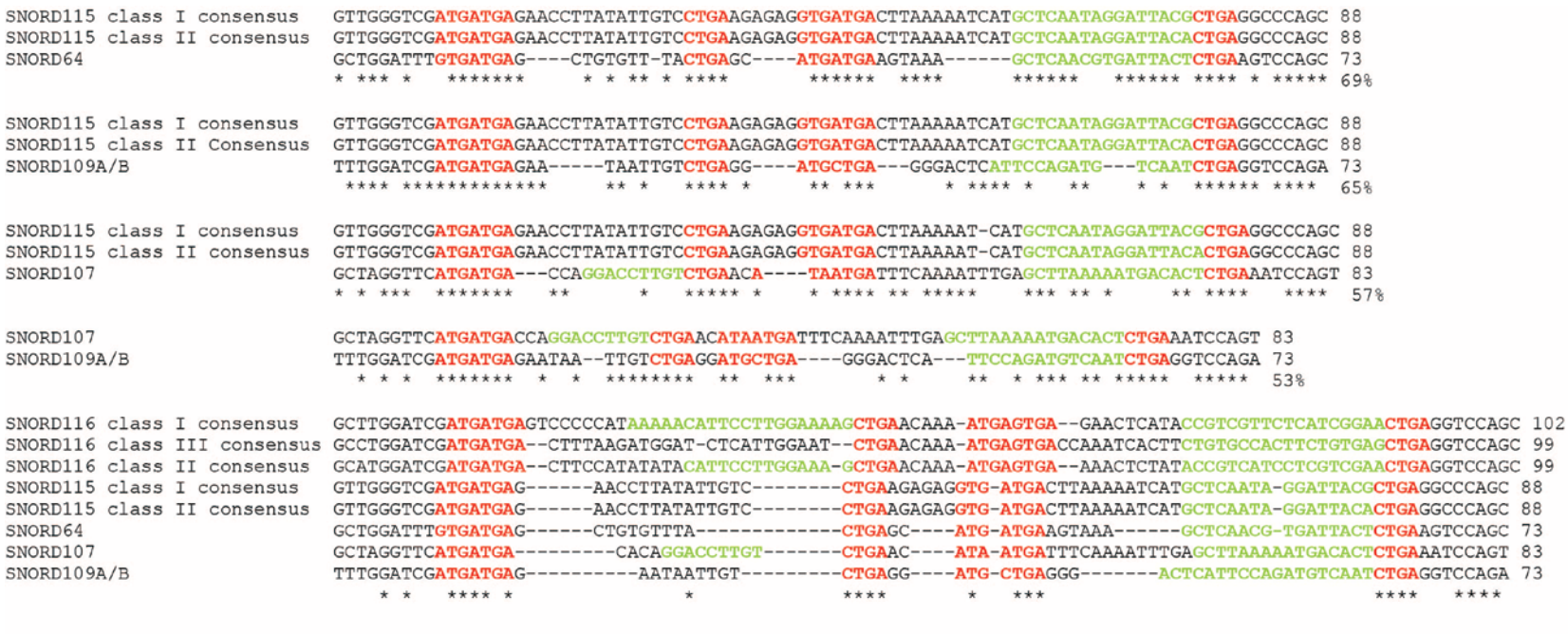


Figure 2.17. Comparison of five classes of PWS C/D box snoRNAs. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

(a)

SNORD64 eutherian orthologs alignment and consensus sequence

```
Chimpanzee                    GCTGGATTTGTGATGAGCTGTGTTTACTGAGCATGATGAAGTAAA-GCTCAACGTGATTACTCTGAAGTCCAGC 73
Sumatran orangutan            GCTGGATTTGTGATGAGCTGTGTTTACCGAGCATGATGAAGTAAA-GCTCAACGTGATTACTCTGAAGTCCAGC 73
Human                         GCTGGATTTGTGATGAGCTGTGTTTACTGAGCATGATGAAGTAAA-GCTCAACGTGATTACTCTGAAGTCCAGC 73
Rhesus monkey                 GCTGGATTTGTGATGAGCTGTGTTTACTGAGCATGATGAAGTAAA-GCTCAATGTGATTACTCTGAAGTCCAGC 73
Small-eared galago            GTTGGATTTATGATGAGCTGTGTTTAGTGAGCATGATGAAGTAAA-GCTCAATGTGATTACTCTGAAGTCCAGC 73
Gray mouse lemur              GCTGGATTTATGGTGAGCTGTGTTTACTGAGCATAATGAAGTGAA-GCTCAAAATGATTAATCTGAAGTCCAGC 73
Rat                           GCTGGATTAATGATGAGCTGTGTTTACTGAGCATAATGAAATGAC-GCTCAAAATAATTACTCTGAAGTC-AGC 72
Rabbit                        GCTGGATTTATGATGAGCTGTATTTACTGAGCATAATGAAGGGAG-GCTCCATGTGATTACTCTGAAGTCCAGC 73
Thirteen-lined ground squirrel GCTGGATTTATGATGAGCTATGTTTACTGAGCATGATGAAGTGAA-GCTCAATATGATTACTCTGAGGTCCAGC 73
Western European hedgehog     GCTGGATTTATGATGAGCTATGTTTACTGAGCATGATGAAATGAA-GCTTGATATGAGAACTCTGAGGTCCAGT 73
Mouse                         GCTGGACTTATGATGAGCTATGTTTACTGAGCATGATGAAATGAT-GCTCAAAATGATTACTCTGAAGTC-AGC 72
Nine-banded armadillo         GCTGGATTTATGATGAGATATGTTTACTGAGCCTGATGAAAAGAAAGCTCACTGTGATTACTCTGAAGTCCAGC 68
Cat                           ACTGGATTTATGATGAGTTATGTTTACTGAGTATGATGAAGTGAA-ACTCAGTGTGAGTACTCTGAAGTCCAGC 67
                                ******  ** **** * * **** ****  * *****   *   **     *** ************ **

Consensus                     GCTGGATTTRTGRTGAGCTRTGTTTACTGAGCATRATGAARTRAA-GCTCAAHRTGATTACTCTGAAGTCCAGC 73
```

(b)

SNORD107 eutherian orthologs alignment and consensus sequence

```
Rhesus monkey                 TTGGCTAGGTTCATGATGACACAGGACCTTGTCTGAAC-ATAATGATTTCAAAATTTGAGCTTAAAAATGACACTCTGAAATCCAGTCAG 89
Human                         TTGGCTAGGTTCATGATGACACAGGACCTTGTCTGAAC-ATAATGATTTCAAAATTTGAGCTTAAAAATGACACTCTGAAATCCAGTCAG 89
Chimpanzee                    TTGGCTAGGTTCATGATGACACAGGACCTTGTCTGATC-ATAATGATTTCAAAATTTGAGCTTAAAAATGACACTCTGAAATCCAGTCAG 89
Sumatran orangutan            TTGGCTAGGTTCATGATGACATAGGACCTTGTCTGAAC-ATAATGATTTCAAAATTTGAGCTTAAAAATGACGCTCTGAAATCCAGTCAG 89
Dog                           TTGGCTAGGTTCATGATGACATAGGACCTTGTCTGAAC-ATAATGA-TTCAAAATTTGAGCTTAAA--TGACACTCTGAAATCCAGTCA- 85
Small-eared galago            TTGGCTAGGTTCATGATGACATAGGACCTTGTCTGAAC-ATAATGATTTCAAAACTTGAGCTTAAAA-TGACA--CTGAAAGCCAGTCA- 85
Cat                           TTGGCTAGGTTCATGATGACACAGGACCTTGTCTGAAC-ATAATGATTTTAAAATTTGAGCTTAAA--TGACACTCTGAAATCTAGTCA- 86
Thirteen-lined ground squirrel TTGGCTAGGTTCATGATGACACAGGACCTTGTCTGAAT-ATGATGATTTCAAAATTTGAGCTTAAA-TTGACACTCTGAAATCTAGTCAG 88
American pika                 TTGACTAGGTTCATGATGACACAGGACCTTGTCTGAAAATTAATGATTTCAAAATTTGAGCTTAAAA-CGACATTCTGAAATCTGGTCA- 88
Little brown bat              -TGGCTAGGTTCATGATGACACAGGACCTTGTCTGAAC-TTCATGATCTCAAAACATGAGCTTGGATATGTCA--CTGAAATCTAGTCA- 85
Horse                         TTGGCTAGGTTCATGATGATACAGGACCTTGTCTGAAC-AAAGTGATTTCAAAATTTGAGCTTAAAA-TGACATTCTGAAATCCAGTCA- 87
Northern tree shrew           TTGGCTAGGTTCATGATGACACTGGACCTTGTCTGAAA-AAAGTGATTTCAAAATTTGAGCTTAAAA-TAATACTCTGAAATCCAGTCA- 87
European shrew                TTGGCTAGATTAATGATGATACAGGACCTTGTCTGAAT-ATACTGATTTCAAAATTTGAGCTTAAAA-TAATATTCTGAAATCCAGTCA- 87
Nine-banded armadillo         TTGGCTAGATTCATGATGACACAGGACCTTGTTTGAAA-ATAATGATATTAAACTTTGAGCTTAAA--CCACACTCTGAAATCCAGTC-- 85
Western European hedgehog     TTGGCTAGATTCATGATGACATTGGACCTTGTATGACT-AAAATGACATCAAAATTTGAGCTTAGAA-TTATACTCTGAAATCCAGTCA- 87
Rabbit                        TTGGCTAGGTTCATGATGACATAGGACCTTGTCTGAATAATAATGATTTCAAATTTTGAGCTAAAG--TGACTTTCTGAAATCCAGTCAG 88
Mouse                         TTGACTGGATTTATGATGATATAGGACCTTGTCTGAAT-ATAATGATTTCAAAAATTGAGCTTACA---GTGA--CTGAAATCTAGTCAA 84
Rat                           TTGACTAGATTTATGATGACATGGGACCTTGTCTGAAC-ACAATGATTTCAAAAATTGAGCTTAGA---GTGACTCTGAAATCTAGTCA- 85
                               ** ** * ** ******* *  ********* ***       ***  * ***   ******             ****** *  ***

Consensus                     TTGRCTAGRTTYATGATGAYAYAGGACCTTGTCTGAAn-AWAATGATTTYAAAAnTGAGCTTAAAAATGACACTCTGAAATCYAGTCAG 89
```

(c)

SNORD109 eutherian orthologs alignment and consensus sequence

```
Human                         TTTGGATCGATGATGAGAATAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 73
Rhesus monkey                 TTTGGATCGATGATGAGAATAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 73
Chimpanzee                    TTTGGATCGATGATGAGAATAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 73
Sumatran orangutan            TTTGGATCGATGATGAGAATAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 73
American pika                 TCTGGATCGATGATGAGAAAAAATGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Cattle                        TCTGGATCGATGATGAGAAAAACTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Rabbit                        TCTGGATCGATGATGAGAACAAGTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Small-eared galago            TCTGGATCGATGATGAGCAAAATTGTCTGAGGACGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Gray mouse lemur              TCTGGATCGATGATGAGCAAAATTATCTGAGGACGGTGAACGACACATTGCAGATGTCAATCTGAGTTCCAGA 71
European shrew                TCTGGATCAATGATGAGAAAAATTGTCTGAGGACGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Horse                         TCTGGATCAATGATGAGCATAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Nine-banded armadillo         TCTTGATCAATGATGAGCATAATTGTCTGAGGATGCTGAGGGACTCATTCTAGATGTCAATCTGAGGTCCAGA 69
Domestic guinea pig           TCTGGATCAATGATGAGCACAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
Dog                           TCTGGATCAATGATGAGCACAATTGTCTGAGGATGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 71
African savanna elephant      TCTGGGTCAATGATGAACATAAATGTCCGAGGATGCTGAGTGACTCATTGCAGATGTCAATCTGAGATCGAGA 71
                              * * * ** *******    ** * ** ***** * ***  *** **** ************** ** ***

Consensus                     TYTGGGTCRATGATGAGMAnAATTGTCTGAGGAYGCTGAGGGACTCATTCCAGATGTCAATCTGAGGTCCAGA 73
```

Figure 2.18. Multisequence alignments of *SNORD64*, *SNORD107*, and *SNORD109*. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

Figure 2.19. Comparison of *SNORD119N* to five classes of PWS C/D-box snoRNAs. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

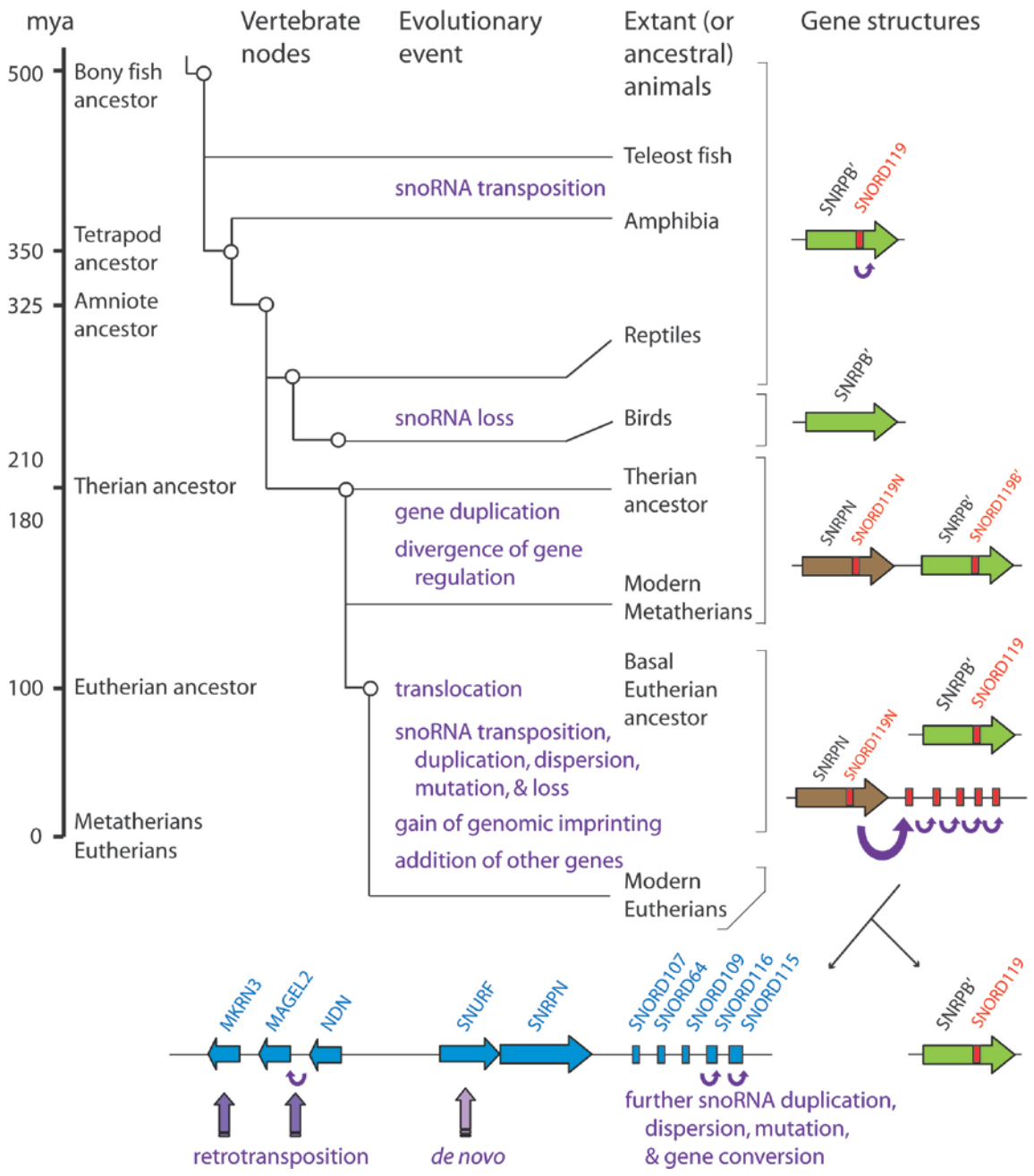


Figure 2.20. Evolutionary model for the origin of the PWS imprinted domain.

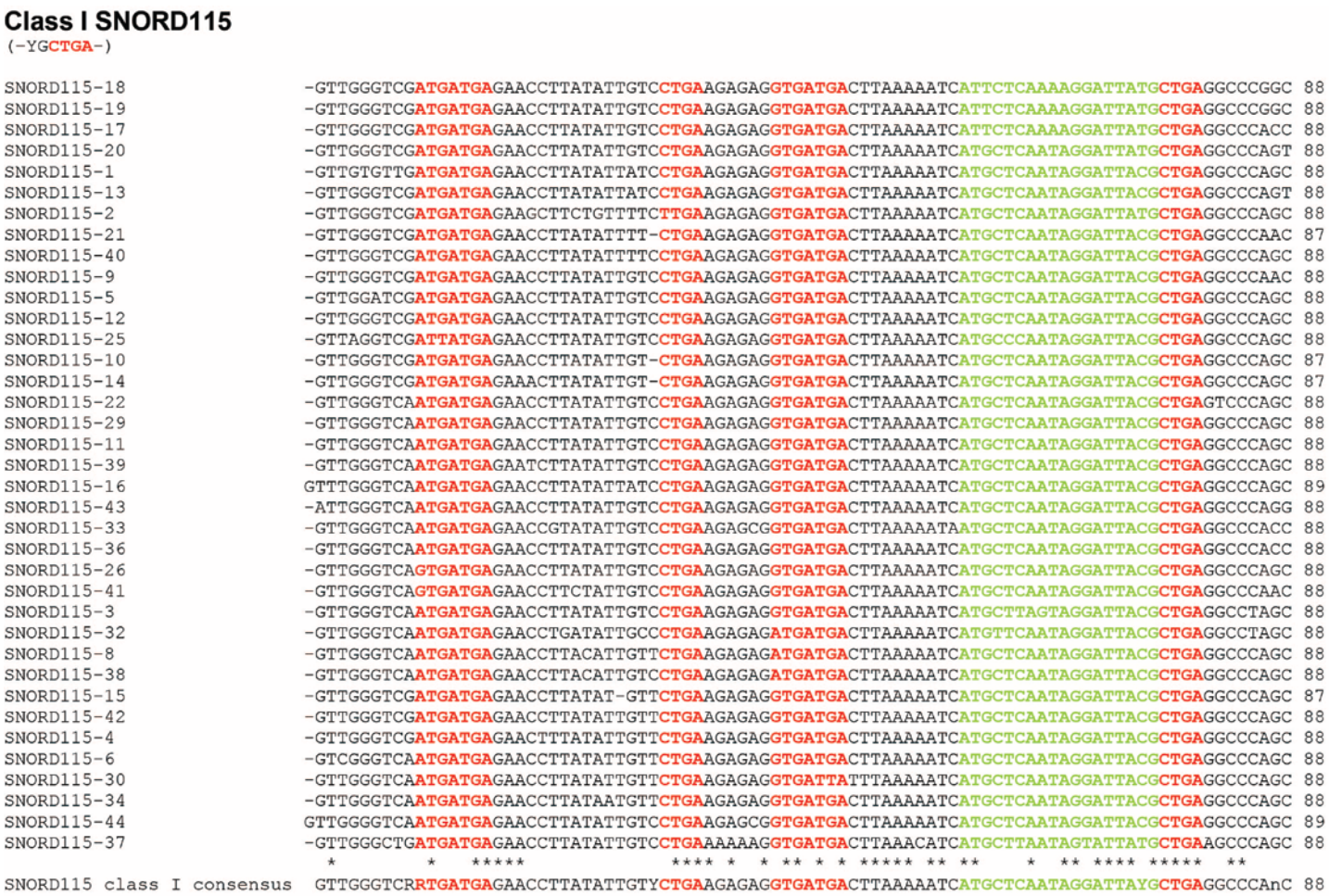


Figure 2.21. (continued below)

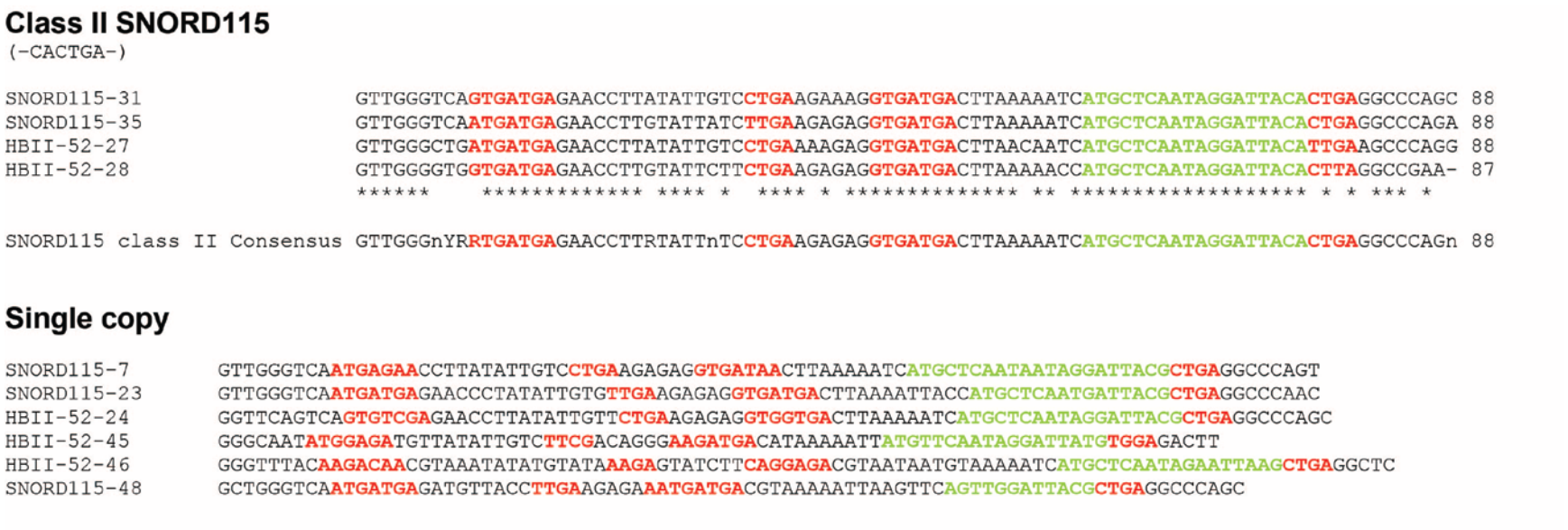


Figure 2.21. Human *SNORD115* family sequence alignment and consensus sequence of each class. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

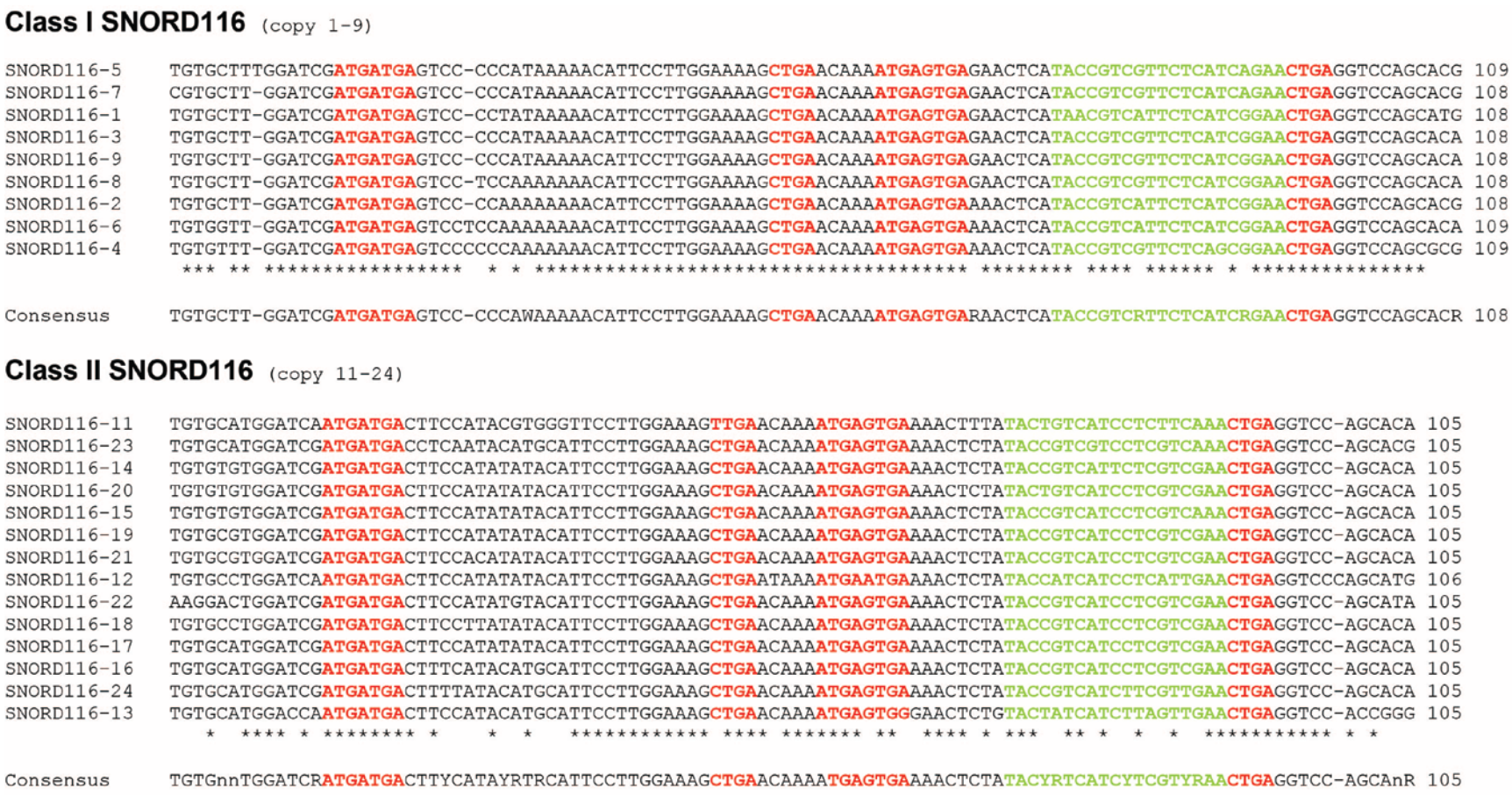


Figure 2.22. (continued below)

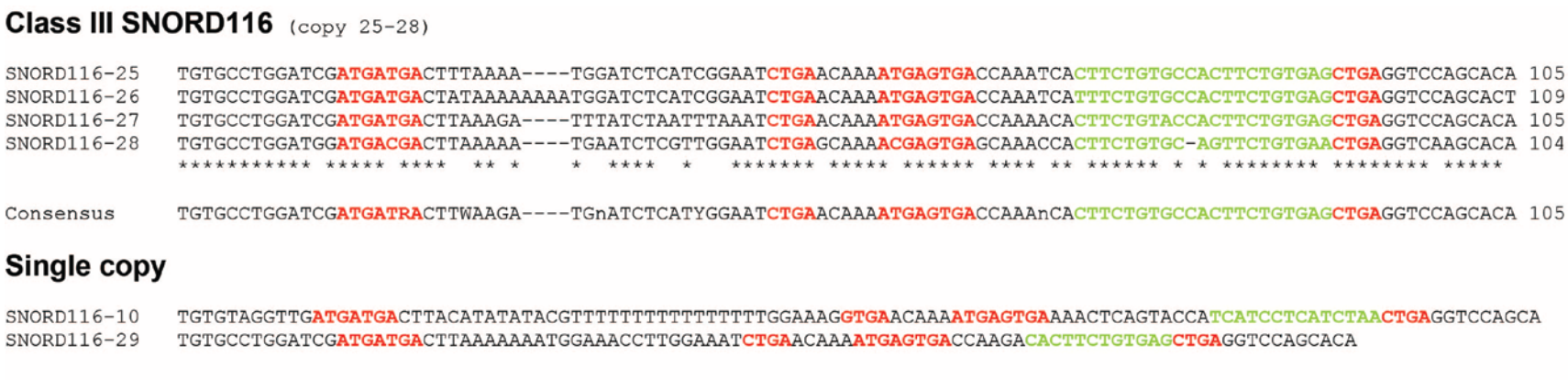


Figure 2.22. Human *SNORD116* family sequence alignment and consensus sequence of each class. Box C/C' and box D/D' are highlighted in bold red: from 5' to 3', these are box C, box D', box C' and box D, respectively. The antisense box is highlighted in bold green and is 5' of box D.

2.4 DISCUSSION

2.4.1 NRF1 regulation of the PWS imprinted domain in somatic cell hybrids

By ChIP assays, we have confirmed the active and repressive chromosomal domains in GM11715, A15 and A15-1 cells. These data showed weak binding of NRF1 to the "NRF1 cluster" in these three somatic cell hybrids. Since our goal was to use a somatic cell hybrid with strong binding of NRF1 to the "NRF1 cluster" in the single copy of human chromosome 15 to study NRF1 regulation on the PWS region, these results indicated none of tested cell lines would be ideal for the "gene targeting" study of "NRF1 cluster". However, my data provided a valuable resource of chromatin information on these three somatic hybrid cell lines for other work in our laboratory.

2.4.2 Evolutionary insights and relationships of the PWS *SNRPN* imprinted locus and snoRNAs

In this study, we reported novel snoRNAs in the *SNRPN* and *SNRPB'* genes of *M. domestica*, *SNORD119N* and *SNORD119B*, respectively, and suggested that by gene duplication of *SNRPB' to SNRPN, SNORD119N* was retained and duplicated from *SNORD119B* in intron 5 of *SNRPB'*. In eukaryotes, functional related genes are in clusters and can be derived from tandem duplication of an ancestral gene (Spitz & Duboule, 2006). Synteny between two species indicates the gene regions have been evolved from a common ancestral gene region (Tanaka-Fujita *et al*.,

2007). For snoRNAs, duplication of a host gene can generate snoRNA paralogs (Weber, 2006, Zemann *et al*., 2006). Combining the sequence similarity of *M. domestica SNORD119N* versus each of the five classes of PWS C/D-box snoRNA genes, the sequence similarity among the five classes of PWS C/D-box snoRNAs, and the syntenic regions of *SNRPN* and *SNRPB'* between human and *M. domestica*, we concluded that *SNORD119N* in *M. domestica* is derived from the same common ancestral snoRNA as for the five classes of PWS C/D-box snoRNA genes by multiple rounds of DNA duplications (**Figure 2.19**). Similar DNA duplication events have been found with multiple copies of U1 promoters in the PWS region (Färber *et al*., 1999). In our analysis, *SNORD119N* is more related to *SNORD64* and *SNORD115* (**Figure 2.18**). This suggests either a more recent evolutionary origin of these snoRNAs or possibly retention of similar functions. Nevertheless, the large number of duplication and diversification events in forming the eutherian PWS-region snoRNAs suggests dynamic evolutionary processes operate on this domain and have selected for evolutionarily. It will be necessary to determine the function of each of the PWS-region snoRNAs in order to understand what evolutionary forces might have driven these processes.

Because *SNURF-SNRPN* is associated with the imprinting control region in the PWS region, our phylogenetic study on the origin of *SNRPN* will have important implications in understanding gene functions and the imprinting mechanism in the PWS region. The comparison of eutherian genomes and the metatherian genome (*M. domestica*) is highly effective in recognizing genes and regulatory sequence evolution. Compared to the *M. domestica* genome, higher mammals have evolved by modification of epigenetic mechanisms instead of primarily inventing new genes (Mikkelsen *et al*., 2007; Renfree, 2007). Genomic imprinting has been found in both eutherians and marsupials, but seems to have a different imprinting mechanism in

marsupials. To better understand why and how imprinting was established in phylogeny, studies on the imprinting mechanism in marsupials become critical. It has been described that the origin of *SNRPN* was derived from gene duplication of an ancestral *SNRPB'* gene, and the expression of brain-specific-imprinted SmN could be compensated by a feed-back loop of SmB/B' expression in order to maintain stoichimetric levels of spliceosomal components (McAllister *et al*., 1989; Gray *et al*., 1999b; Matera *et al*., 2007). The indispensability of SmB'/B/N was consistent to our finding that *SNRPB'* existed in all species (data not shown), while *SNORD119* existed in most species we analyzed, including all mammals, one amphibian, reptiles, and two kinds of fish. The constrained sequence of *SNRPB'* and *SNORD119* implied the functional importance of both genes.

In this study, we also predicted the function of *SNORD119* is to target 28S rRNA at position 4560 for 2'-*O*-methylation based on sequence alignment and thermodynamic calculation (**Figure 2.14; Figure 2.15**). Methylation modification of rRNA on position 4560 has been experimentally confirmed in human and *X. laevis*, but was not found in chicken (Khan & Maden, 1976; Maden & Hughes, 1997, listed as position 4550). Consistent with this, *SNORD119* was found in *SNRPB'* of human and *X. laevis*, but not in chicken.

Considering evolutionary explanation for the snoRNA copies in PWS region, it can be traced by the sequence similarity between *M. domestica SNORD119N* and each of the five classes PWS C/D-box snoRNAs. First, PWS C/D-box snoRNAs were derived from *SNORD119N* by a series of DNA duplications after which they gained new function in brain neonatal and/or postnatal development. This is supported by recent studies that snoRNAs can also target pre-mRNA modification for gene silencing and regulation (Zhao & Yu, 2008) and *SNORD115* can target serotonin 2C receptor to regulate gene expression (Kishore & Stamm, 2006). There is a

growing number of snoRNA found to have tissue-specific expression pattern, most probably reflecting that of the host gene (Cavaillé *et al*., 2000; Runte *et al*., 2001). A PWS patient was found to have distal *SNURF-SNRPN* translocation breakpoint with no gene expression of *SNORD116*, *IPW* and *PAR1* (Wirth *et al*., 2001). A mouse model with a paternal deletion from *Snrpn* to *Ube3a* showed a phenotype of hypotonia, growth retardation, and partial lethality (Tsai *et al*., 1999) that is similar to the neonatal presentation of PWS and other PWS mouse models. These data suggest that the loss of PWS C/D-box snoRNAs contributes to the disease of PWS (Cavaillé *et al*., 2000; Runte *et al*., 2001). Another recent study on a paternally-inherited *Snord116* deletion mouse model showed that snoRNA deletion in the PWS region was not lethal, but led to growth deficiency and a mild hyperphagia (Ding *et al*., 2008) albeit the animals weighed less than control littermates. All these studies suggest there is some specific function of PWS C/D-box snoRNAs in neonatal to postnatal development. Alternatively, the PWS C/D-box snoRNAs that have high sequence similarity to *SNORD119N*, such as *SNORD64* and *SNORD115*, may target the same position of 28S rRNA in the brain. For example, in our analysis, we predicted that PWS C/D-box snoRNAs *SNORD115-3* could alternatively target the same position of 28S rRNA (**Figure 2.15**) during postnatal development for brain-specific function (Cavaillé *et al*., 2000; Runte *et al*., 2001; de los Santos *et al*., 2000).

Additional analysis, in our studies, implied some of the copies in *SNORD115* and *SNORD116* may not be functional because of mutations in the box-C/C' and box-D/D' regions (**Figure 2.21 & 2.22**), the gene structure of which were similar to the pseudogene *HBII-437* (Runte *et al*., 2001).

To summarize, our study identified the origin of the five classes PWS C/D-box snoRNAs by gene duplication and diversification from an ancestral host gene with its intronic snoRNA.

Clearly, snoRNA evolution is labile, with duplication, transposition, and loss being detected in different phylogenetic lineages. It remains unclear why multiple copies of PWS C/D-box snoRNAs exist. It may be due to dosage requirement in specific period of development and/or functional compensation to some loss-of-function copies, such as the ones in the classes of *SNORD115* and *SNORD116.* In the long term, functional studies on the five classes of PWS C/D-box snoRNAs will allow these questions to be addressed, which will also shed light on the role of the box C/D-snoRNAs in disease mechanisms of PWS.

3.0 REGULATORY ROLES AND MECHANISMS OF NRF1 IN CIRCADIAN RHYTHMS

3.1 INTRODUCTION

Circadian rhythms, an "internal body clock", govern the metabolic, hormonal, physiological, and behavioral pace of an organism in a 24-hour period, and exist from lower organisms such as bacteria to higher mammals such as human (Roenneberg & Merrow, 2005). The central mammalian circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus is entrained by light acting on retinal neurons that project to the SCN, and uses neuronal and hormonal mechanisms to coordinate clocks in organs throughout the body (Maywood *et al*., 2007; Takahashi *et al*., 2008). In addition to the SCN, peripheral organs, such as liver and heart, have circadian rhythms that receive output signals from the SCN (Rutter *et al*., 2002; Hastings *et al*., 2007) and additionally can be entrained by food or energy balance (Mendoza, 2007).

At the cellular level, circadian rhythms are comprised of positive and negative feedback loops. In this feedback system, the CLOCK and BMAL1 polypeptides form a heterodimeric complex which binds to an E-box (5'-CANNTG-3') transcriptional element and functions as a transcriptional activator in the circadian pacemaker to regulate clock-controlled genes (CCGs, Steeves *et al*., 1999; Gekakis *et al*., 1998; Kondratov *et al*., 2003). In contrast, PER and CRY

polypeptides also form heterodimers and mediate the negative limb of the circadian transcriptional feedback loop (Sun *et al*., 1997; Darlington *et al*., 1998; Hastings *et al*., 2007; Brown *et al*., 2005). The increase of PER/CRY heterodimers inhibits CLOCK/BMAL1 function. Subsequently, PER and CRY are degraded and cycling of this system generates the circadian oscillations (Reppert & Weaver, 2002). Additionally, *NR1D1* forms an ancillary loop to coordinate the pacemaker (Takahashi *et al*., 2008) regulating *BMAL1* via *REV-ERBα* response elements (ROREs, Preitner *et al*., 2002; Ueda *et al*., 2002) and also to repress itself by autoregulation (Adelmant *et al*., 1996). The fine-tuned regulation of both loops leads CCGs regulated by CLOCK/BMAL1 to act under circadian control. Although many key regulatory genes in the circadian system have been found, there may still be some missing factors that coordinate the system.

It has been suggested that *DBP*, a key clock output gene, plays a role in the feedback system for circadian rhythms (Takahashi *et al*., 2008). *DBP* contributes to activation of *PER1* by binding to the *PER1* promoter (Yamaguchi *et al*., 2000). Expression of *DBP* is driven by oscillations of chromatin structure that regulate the binding of CLOCK/BMAL1 to E-box motifs within enhancer regions located in the first and especially the second intron (Ripperger *et al*., 2000; Ripperger & Schibler 2006). Indeed, deletion of two E-box motifs in intron 2 abolished the oscillation of *DBP* expression and chromatin structure in Rat-1 cells and in a mouse model (Ripperger *et al*., 2000; Ripperger & Schibler 2006). In this work, we identify a conserved NRF1 motif within the intron 2 enhancer element of *DBP*. Furthermore, we identify conserved NRF1 binding sites in the 5' regulatory elements of 25 of 45 circadian regulatory genes, including *CLOCK*, *CRY1*, *PER1*, and *NR1D1*. These are functional binding sites as shown by ChIP, expression studies following siRNA knockdown of *NRF1* mRNA levels, and transient

transfection assays of wildtype and mutant promoter and promoter-enhancer luciferase reporter constructs. Further, co-immunoprecipitation shows that NRF1 and the phosphorylated, active form of CLOCK interact in a molecular complex. In serum-induced NIH3T3 cells with circadian oscillations of *Dbp* and *Nr1d1* mRNA, *Nrf1* mRNA and protein levels show ultradian oscillations. Based on these results, we propose that NRF1 has a major role in activating expression of circadian regulatory genes from both the positive and negative feedback regulatory loops, and consequently is positioned to integrate many aspects of circadian biology.

3.2 MATERIALS AND METHODS

3.2.1 Bioinformatics and phylogenetic analyses

Genomic sequences for *CLOCK*, *PER1*, *CRY1*, *DBP*, *NR1D1*, and 45 other circadian regulatory genes were obtained by NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with the human genes from Ensembl database. Sequences spanning each gene were exon-masked and/or Repeatmasked [using the "human" in "DNA source" option] and used for BLAST. The consensus NRF1 recognition sequence "YGCGCANGCGCR" were used for the search and allowed 1 nucleotide change in either "GCGC" arm. Putative NRF1 binding sites were searched 5 kb upstream of TSS and at least 1 kb into intron 1 of 45 reported circadian regulatory genes. For *CLOCK*, *PER1*, *CRY1*, *DBP*, and *NR1D1*, all sequence was transformed into FASTA format on ReadSeq (http://searchlauncher.bcm.tmc.edu/seq-util/readseq.html) and the alignments were performed by Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Alignments were adjusted as needed to maximize parsimony.

3.2.2 Gene expression studies

RNA was extracted from SK-N-SH human neuroblastoma cells and NIH3T3 mouse embryonic fibroblast cells, and reversed transcribed into cDNA using SuperScript® III reverse transcriptase according to the manufacturer's instruction (Invitrogen). Regular PCR was performed to test gene expression of 25 circadian regulatory genes using the primers in **Table 3.1**.

Table 3.1. RT-PCR primers for circadian gene expression studies (human SK-N-SH cells).

Target gene (primer)[a]	Primer number	Exon	Primer sequences
CLOCK (F)	RN3519	17	5′-GATAATCGTATAAACACAGTCAGTC-3′
CLOCK (R)	RN3520	17-18	5′-GGTGTTGAGGAAGGGTCTGAG-3′
PER1 (F)	RN3521	16	5′-GTCTCTGTGGGGACCAAGAAAG-3′
PER1 (R)	RN3522	17	5′-ACTGGCTCCTTCCGTGGGGTG-3′
CRY1 (F)	RN3523	8	5′-CTGTTGGTTTTGGTAGGAGAAC-3′
CRY1 (R)	RN3524	9	5′-ATTTTGCAGGGAAGCCTCTTAG-3′
NR1D1 (F)	RN3525	6	5′-CTTCCGTGACCTTTCTCAGC-3′
NR1D1 (R)	RN3526	7	5′-GCAAAGCGCACCATCAGCAC-3′
WDR5 (F)	RN3527	12-13	5′-TCTCTGTTACTGGTGGGAAGTGG-3′
WDR5 (R)	RN3528	13-14	5′-TGTTGAGATCACGACATCTGTGTG-3′
TIMELESS (F)	RN3529	12-13	5′-GCTGGCAACAGTGAATGAGATG-3′
TIMELESS (R)	RN3530	13	5′-CAGGTCACGAAGGAAAGAGC-3′
DBP (F)	RN3531	3-4	5′-GAGGAGCAGAAGGATGAGAAATAC-3′
DBP (R)	RN3532	4	5′-CGCACCGATATCTGGTTCTCC-3′
FBXL15 (F)	RN3533	3	5′-CAGAACTCCACCACCTTGACC-3′
FBXL15 (R)	RN3534	4	5′-AACGCAGCACGGGGCAGTAC-3′
ALAS1 (F)	RN3535	3	5′-GAGGATGTGCAGGAAATGAATG-3′
ALAS1 (R)	RN3536	4	5′-CCCTCCATCGGTTTTCACACT-3′
HSF1 (F)	RN3537	5	5′-CATGCCCAGCAACAGAAAGTCG-3′
HSF1 (R)	RN3538	6	5′-CTGCACCAGTGAGATCAGGAAC-3′
CHORDC1 (F)	RN3539	10-11	5′-GGGTGTGATTGATGTAAAGCG-3′
CHORDC1 (R)	RN3540	11	5′-CTGCATCGGTTCAGCTTTTCTC-3′
NPAS2 (F)	RN3541	3	5′-CCCCAAGAGTTACCTGTCCC-3′
NPAS2 (R)	RN3542	4	5′-AGGCAGGAGCTGCTGTGTGAG-3′
ARNTL (F)	RN3543	12	5′-GTGAACGGGGAAATCAGGGTG-3′
ARNTL (R)	RN3544	12-13	5′-TAGCTGTTGCCCTCTGGTCTAC-3′
ARNTL2 (F)	RN3545	9	5′-ATGGATGCTTACCCAACTCAAAG-3′
ARNTL2 R	RN3546	10	5′-CCAGCTTCTCAAGTAACCAGTG-3′
CRY2 (F)	RN3547	8	5′-GTTCTTCCACTGCTACTGCCC-3′
CRY2 (R)	RN3548	8-9	5′-AGGTATCGCCTGATGTAGTCCC-3′
PER2 (F)	RN3549	10	5′-CCTCTCCTGGGCTACCTACC-3′
PER2 (R)	RN3550	10-11	5′-GCAGGATCTTTTTGTGGATGGC-3′
PER3 (F)	RN3551	15	5′-ATAAGCCAATGCGGTTACAGC-3′
PER3 (R)	RN3552	16	5′-GTCCAGGGCTCACAGAAGAG-3′
BHLHB2 (F)	RN3553	4	5′-GTACCTGAATAAGAAGACAACTC-3′
BHLHB2 (R)	RN3554	4-5	5′-CACATCCAAAGTTCCTTCTGTAC-3′
BHLHB3 (F)	RN3555	1-2	5′-AGATTTTATAGGACTGGACTATTC-3′

Table 3.1. (continued)

Target gene (primer)[a]	Primer number	Exon	Primer sequences
BHLHB3 (R)	RN3556	2	5′-TGTCGTCTCGTTTCATGCTCC -3′
NONO (F)	RN3557	5	5′-AAGAGGGACTTCCAGAGAAGCTG-3′
NONO (R)	RN3558	5-6	5′-GGGTGGCTGCTCTCGTTCCTTG-3′
CSNK1E (F)	RN3559	7	5′-GCACATCGAGAGCAAGTTCTAC-3′
CSNK1E (R)	RN3560	8	5′-GTTGTAGTCGCCCTCAGCTC -3′
FBXL3 (F)	RN3561	3	5′-AACACTTGGACTTATTTCAACTGC-3′
FBXL3 (R)	RN3562	4	5′-GAGTTTACGAACACAACTGTCAG-3′
CIPC (F)	RN3563	2	5′-CTTGGGATGGCTGCTGCTGAG-3′
CIPC (R)	RN3564	3	5′-GGTGTTGAGGAAGGGTCTGAG-3′
EZH2 (F)	RN3565	10	5′-AAACGGGGGGAGAGAACAATG-3′
EZH2 (R)	RN3566	10-11	5′-ACCGAGAATTTGCTTCAGAGG-3′
PPARGC1A (F)	RN3567	11-12	5′-AGTAAATCTGCGGGATGATGGAG-3′
PPARGC1A (R)	RN3568	12	5′-TTCAAGAGCAGCAAAAGCATCAC-3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

3.2.3 Chromatin immunoprecipitation (ChIP)

Approximately 1×10^6 SK-N-SH cells were plated on 35 cm^2 plates with regular media [Alpha modified MEM + 10% Fetal Bovine Serum (FBS) + 1% penicillin-streptomycin (P/S) + 1% of 100 mM L-glutamine + 1% nonessential amino acid + 1% sodium pyruvate]. When cells reached 70-80% confluence, formaldehyde was added to the media to make a final concentration of 1% and the plates were incubated at 37°C for 10 minutes to cross-link proteins and DNA. After washing with 5 ml ice-cold PBS, cells were scraped from the plates in 5 ml ice-cold PBS using a cell lifter, put into a 15 ml tube, centrifuged, re-suspended in SDS lysis buffer (ChIP assay kit, Millipore) and sonicated to shear DNA. Samples were precleared with protein G-agarose/salmon sperm beads (Millipore). Protein-DNA complexes were immunoprecipitated with anti-NRF1 (from Dr. Daniel Raines), anti-H3K4me1 [Histone H3 (mono methyl K4) antibody, Abcam, Catalog #: ab8895], anti-H3K4me3 [Anti-trimethyl-Histone H3 (Lys4), Millipore, Catalog #: 07-473], or anti-H3K9me2 [Anti-dimethyl-Histone H3 (Lys9), Millipore, Catalog #: 07-521]. Complexes were collected with Protein G agarose/salmon sperm beads and washed. Protein-DNA complexes were eluted off the beads and cross-links were reversed by incubation with NaCl at 65ºC overnight. On the next day, DNA was recovered by phenol-chloroform extraction and precipitated by ethanol. PCR was performed using the recovered immunoprecipitated DNA as templates and primers from **Table 3.2**. The PCR products were run on an agarose gel to examine the results.

Table 3.2. PCR primers for ChIP assays of circadian gene *cis*-regulatory elements, in human SK-N-SH cells or mouse Neuro2a cells.

Target gene (primer)[a]	Primer number	Primer sequences
CLOCK (F)	RN3377	5′-GCCTGCAGAGCCAGATTTCG-3′
CLOCK (R)	RN3378	5′-AAGCCGAGTCCGTGATTGCG-3′
CLOCK intron 1 (F)	RN3379	5′-GAGTGCCGGTTGGCTTCTTG-3′
CLOCK intron 1 (R)	RN3380	5′-CACTACCCCGCCTGTGTCCAG-3′
PER1 (F)	RN3381	5′-CAGCTGACGTCGGTTTCCCTG-3′
PER1 (R)	RN3382	5′-GTAGACAAAAACCTGGACACTTCC-3′
CRY1 (F)	RN3383	5′-CTGAAGGAAACCGGACAATTTC-3′
CRY1 (R)	RN3384	5′-GCTGAGACCCGGATGAGCAC-3′
NR1D1 (F)	RN3387	5′-ATCACATGGTACCTGCTCCAGTG-3′
NR1D1 (R)	RN3388	5′-GAAGTAAGTAGGTGATGGGGAGAA-3′
WDR5 (F)	RN3389	5′-CGTAGCGCTCCTCCGAGAG-3′
WDR5 (R)	RN3390	5′-GGACTCAGCTCGACAAGGC-3′
TIMELESS intron 1 (F)	RN3391	5′-GAGTGAGTGTGTGGCGAGAGG-3′
TIMELESS intron 1 (R)	RN3392	5′-GGAGACTAAGGAGCAGAGTACAG-3′
DBP intron 2 (F)	RN3393	5′-CCCTTCCCAGCGGCACATTCC -3′
DBP intron 2 (R)	RN3394	5′-CAGCTAAGGACACAGGTTCAGG -3′
FBXL15 (F)	RN3395	5′-GTCTGTTCGGGCAGTCAGG-3′
FBXL15 (R)	RN3396	5′-CAGACCCCTGCAAAGATAGAG-3′
ALAS1 (F)	RN3397	5′-ATATCCGCAGAGCCCAAGAAG-3′
ALAS1 (R)	RN3398	5′-CACTCAAGTCGAGAAGTCCAAAC-3′
CHORDC1 (F)	RN3401	5′-CTGTTGTCTGGGCTCCACTTC-3′
CHORDC1 (R)	RN3402	5′-ACGATCCGTTGCGTTTCAGG-3′
CIPC (F)	RN3403	5′-GACGACGAGGTTGTCATGGTG-3′
CIPC (R)	RN3404	5′-CTCCCCTACTTCCACCTCCTTTC-3′
EZH2 (F)	RN3405	5′-GCGTCCCTTACAGCGAACCC-3′
EZH2 (R)	RN3406	5′-CAATCGCCATCGCTTTTATTTG-3′
HSF (F)	RN3411	5′-GCACAGCCGCTTCCGACAG-3′
HSF (R)	RN3412	5′-GCGCGTGTTGGTCCCACC-3′
TEF (F)	RN3598	5′-CGCCAATCAGGGGACACAG-3′
TEF (R)	RN3599	5′-CGCCTCCTTCCATCGCTAC-3′
PPP5C (F)	RN3600	5′-TCTCATTCCCTATTGGCTCTC-3′
PPP5C (R)	RN3601	5′-GCCGCACAAGTGTCGTAAAG-3′
BTRC (F)	RN3602	5′-CGTAGCCTCAGTTTTTTTCCTG-3′
BTRC (R)	RN3603	5′-CGCCCTCTCTTACCTCTCAG-3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index

and http://www.ensembl.org/Mus_musculus/Info/Index). Unless indicated otherwise, all PCR primers are located in the promoter of the indicated gene.

3.2.4 *NRF1* siRNA assays

NRF1 siRNA primers were designed (Brummelkamp *et al*., 2002; Smith *et al*., 2004), cloned into the pSUPER siRNA vector and then sequenced to confirm the correct structure. SK-N- SH cells were sub-cultured onto 75 cm^2 flasks 1 day before the nucleofection and reached 80-90% confluence. On the day of nucleofection, 4 μg of pSUPER-NRF1 DNA were transfected into 1×10^6 SK-N-SH cells using an Amaxa cuvette (Lonza) with Nucleofector® (Lonza) and the transfected cells were plated onto a 6-well plate. 24 hr after nucleofection, RNA was extracted with Trizol and treated by DNase I to remove genomic DNA. 1 μg of RNA from each sample was reverse transcribed into cDNA using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacture's instructions. RT-PCR was performed using primers for each gene (**Table 3.1**) to examine gene expression with *GAPDH* as a negative control and *NRF1* as a positive control to ensure effective down-regulation by siRNA.

3.2.5 Reporter constructs and luciferase reporter assays

Based on sequence alignments of conserved promoter sequence of *CLOCK*, *PER1*, *CRY1*, *NR1D1* and *DBP*, and enhancer sequence of *PER1* and *DBP*, luciferase reporter constructs were made by PCR cloning using primers in **Table 3.3** to clone the minimal promoter (all five genes) and enhancer (*PER1* intron 1 and *DBP* intron 2) sequences using genomic DNA from human SK-N-SH cells as templates. PCR products were digested with *Bgl*II and *Hin*dIII for promoters or

*Bam*HI and *Sal*I for enhancers, and ligated products directionally into the pGL3basic vector (Promega). For the *CLOCK* promoter, *NR1D1* promoter, and *DBP* enhancer, site-directed mutations in the putative NRF1 binding sites were performed using primers in **Table 3.3** with a GeneTailor™ Site-Directed Mutagenesis Kit (Invitrogen) according to the manufacturer's instruction and the mutated products were amplified by Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). However, by sequence analysis, we found four unexpected mutations, which were introduced by PCR, in either the promoter or in the luciferase gene of the *DBP*-pr+enh-NRF1 construct, and one mutation in the luciferase gene of the *CLOCK*-pr-mu construct. To correct these errors, *Bam*HI and *Sal*I were used to isolate the desired *DBP* enhancer fragment carrying the NRF1 site mutation, while *Bgl*II and *Hin*dIII were used to isolate the desired *CLOCK* promoter fragment with the NRF1 site mutation, and each used to generate the expected constructs, respectively. Both new constructs were sequenced to confirm correct insertions. To make the NRF1 site mutation in the putative *PER1* enhancer, a minigene was designed (**Table 3.3**) and synthesized by Integrated DNA Technologies, Inc. The mutated *PER1* enhancer fragment was released from the synthetic construct and ligated into the *PER1*-pr following *Bam*HI and *Sal*I digestion. Subsequently, five groups of constructs were transfected into SK-N-SH human neuroblastoma cells in a 6-well plate or NIH3T3 cells in a 24-well plate using Lipofectamine 2000 (Invitrogen). For each well, 1 μg of pGL3basic-derived vector containing a firefly luciferase gene was co-transfected with 25 ng of pRL-SV40 vector containing a *Renilla* luciferase gene. Cells were lysed 24 hr after transfection by passive lysis buffer (Promega). Luciferase activity was measured using a Dual-luciferase reporter assay (Promega) on a Luminometer $20/20^n$ device (Turner BioSystems). For each sample, the firefly luciferase activity was normalized to *Renilla* luciferase activity. The relative luciferase activity was normalized to pGL3basic vector luciferase

activities. Each experiment was repeated three times to collect data for statistical analysis and analyzed by a two-tailed t-test.

Table 3.3. PCR primers and a minigene used for luciferase constructs and mutagenesis.

Target gene (primer or oligonucleotide)[a]	Primer number	Primer sequences
CLOCK promoter (F)	RN3644	5′-GACT**AGATCT**GCGGCTCCGTGCTGCCTAAC-3′
CLOCK promoter (R)	RN3645	5′-GAAA**AAGCTT**CAAGCCGAGTCCGTGATTG-3′
DBP promoter (F)	RN3647	5′-CACA**AGATCT**ATCAGGCAGCACGAGCAGAGC-3′
DBP promoter (R)	RN3648	5′-GGTT**AAGCTT**CCAAAGCAAACTTCTTTCGC-3′
DBP enhancer (F1)	RN3649	5′-CTTT**GGATCC**CGGCACATTCCTGCGCCACG-3′
DBP enhancer (F2)	RN3650	5′-CAAT**GTCGAC**CTAAGGACACAGGTTCAGG-3′
DBP enhancer (R1)	RN3651	5′-CTTT**GGATCC**TGAGTCCTCCCATCCCTCCTC-3′
DBP enhancer (R2)	RN3652	5′-CAAT**GTCGAC**TGCTGACGCCTGCTTCCCTC-3′
NR1D1 promoter (F)	RN3653	5′-CTTT**AGATCT**CGCAGTCCGCCCACTTTGTC-3′
NR1D1 promoter (R)	RN3654	5′-CTTT**AAGCTT**CTTTTGCCCGAGCCTTTCCCTG-3′
PER1 promoter (F)	RN3655	5′-CACA**AGATCT**CTCTTCAGCCCAGCACCAGC-3′
PER1 promoter (R)	RN3656	5′-CTTT**AAGCTT**TCCACCGGGCGGGCGGGGAG-3′
PER1 enhancer (F)	RN3657	5′-TTAA**GGATCC**CGTTCCCCCACTTCCGCCG-3′
PER1 enhancer (R)	RN3658	5′-CACA**GTCGAC**CTCCAGGCTAATTTTATC-3′
CRY1 promoter (F1)	RN3659	5′-CTTT**AGATCT**CTTACCCTCTGGAACGCAGC-3′
CRY1 promoter (F2)	RN3675	5′-CTTT**AGATCT**CAAGCTCCCCGCATCCCC-3′
CRY1 promoter (R1)	RN3660	5′-CCAT**AAGCTT**TGTTTACTACACTGGCTCGG-3′
CRY1 promoter (R2)	RN3661	5′-CCCT**AAGCTT**CCTCGGCCCCGCCCCCG-3′
CLOCK promoter mutation (F)	RN3693	5′-GTGCTGCCTAACGGGGCAAGT***aaa***AT***aaa***CACCGAGCCG-3′
CLOCK promoter mutation (R)	RN3852	5′-ACTTGCCCCGTTAGGCAGCACGGAGCCGCGC-3′
DBP enhancer mutation (F)	RN3676	5′-CTCCCATCCCTCCTCCGCGCTTCGC***aa***AG***aaa***CCTTCAAGCG-3′
DBP enhancer mutation (R)	RN3677	5′-GCGAAGCGCGGAGGAGGGATGGGAGGACTCACGTGG-3′
NR1D1 promoter mutation (F)	RN3855	5′-TCCCGACAGTCTTGTCGTTG***aaa***AG***aaa***CGCAAGAGCTC-3′
NR1D1 promoter mutation (R)	RN3856	5′-CAACGACAAGACTGTCGGGATTTGTAGTCCACC-3′

Table 3.3. (continued)

Target gene (primer or oligonucleotide)[a]	Primer number	Primer sequences
PER1 enhancer mutation minigene (sense-strand only is shown)	RN3717	5′- TTAA**GGATCC**CGTTCCCCCACTTCCGCCGGGAAATGG GGGAGGGGTCGCTCCTCCCGCCCTCCTGTGGTCCCTC CAGCAACCGCTGAGCTCAGCAGCTGACGTCGGTTTCC CTGGCGACCGTGGCGGCGGCGGAAGCGCGTGGTGGGG CCGCGCACGTCGGCG***aaa***AT***aa***GCAGCGGGGGTGGCA CCGCCCCCGGATAAAATTAGCCTGGAG**GTCGAC**TGTG -3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index). For primer sequences, bold and underline represent restriction enzyme sites used for cloning purposes, while bold, italicized lowercase letters represent mutations (shown only for the sense strand of oligonucleotides). Each of the five mutations generated is within an NRF1 site within the corresponding wildtype (WT) DNA sequence.

3.2.6 Luciferase reporter assays on co-transfection of the *DBP* promoter-enhancer luciferase construct and expression vectors for CLOCK, BMAL1 and NRF1

5×10^4 NIH3T3 cells were plated into a 24-well plate a day before the transfection. On the day of the experiment, each well was transfected with 250 ng pGL3b-DBP pr+enh construct, 25 ng pRL vector and/or (1) 125 ng pcDNA3-HA-Clock and 125 ng pcDNA3-HA-BMAL1 (these expression vectors were a gift from Dr. M.P. Antoch, Roswell Park Cancer Institute, Buffalo; Kondratov *et al*., 2006); (2) 125 ng pcDNA3-NRF1-VP16 (a gift from Dr. T. Gulick; Ramachandran *et al*., 2008); and (3) 125 ng of each of the three expression vectors, using

Lipofectamine 2000 for 20 minutes, the medium was changed to 500 µl/well OPTI-Medium. 24 hr after the transfection, cells were lysed by passive lysis buffer (Promega) and luciferase activity was measured by a Luminometer $20/20^n$ device (Turner BioSystems). For each sample, the firefly luciferase activity was normalized to *Renilla* luciferase activity. The relative luciferase activity was normalized to the luciferase activity of the pGL3basic vector. Each experiment was repeated three times to collect data for statistical analysis and analyzed by a two-tailed t-test.

3.2.7 Co-immunoprecipitation

Approximately 2×10^6 NIH3T3 cells were plated in 35 cm^2 plates with regular medium [(Dulbecco's modified Eagle medium (DMEM)+10% calf bovine serum (CBS) + 1% penicillin-streptomycin (P/S)]. When cells reached 80% confluence, media was removed and cells were washed once with 5 ml ice-cold PBS/Phosphatase Inhibitors (Nuclear Complex Co-IP Kit, Active Motif). By adding another 5 ml ice-cold PBS/Phosphatase Inhibitors, cells were removed gently by a cell lifter and collected by centrifugation, and cell pellets were re-suspended in 1 ml 1× Hypotonic Buffer with Detergent and the cells lysed by incubation on ice for 15 min. After centrifugation, pellets (nuclear fraction) were re-suspended in 200 µl Complete Digestion Buffer with 1 µl Enzymatic Shearing Cocktails and incubated at 4°C for 90 min. To stop the reaction, 4 µl 0.5 M EDTA was added to each sample and centrifuged at 14,000 × g at 4°C for 10 min to obtain supernatant. Protein concentration was measured by Bovine Gamma Globulin (BGG) Protein Standards assays (Pierce). Control agarose resin (Cross-linked Co-IP kit, Pierce) was used to pre-clear the cell lysates before immunoprecipitation.

Anti-NRF1 (received from Dr. Daniel Reines) and anti-BMAL1 (N-20, Santa Cruz Biotechnology) were each cross-linked to Protein A/G Plus Agarose on Pierce Spin Column by

DSS solution (Cross-linked Co-IP kit, Pierce). For each co-IP reaction, 500 μg of nuclear extracts were diluted into a total of 600 μl IP Incubation Buffer and incubated overnight at 4°C with rotation. On the next day, the columns were washed and co-IP products were collected by elution buffer according to the manufacturer's instruction. The antigens were then used for western blot analysis. Cell lysates incubated with control agarose resin overnight were used as a control according to the manufacturer's instruction.

3.2.8 Western blot

Immunoprecipitated protein samples were loaded and separated on 4-12% Bis-Tris gels (BioRad, Criterion XT Precast Gel) and transferred onto a nitrocellulose membrane. IP samples by anti-rabbit NRF1 and anti-rabbit BMAL1 were detected on a western blot with anti-CLOCK (S-19, Santa Cruz Biotechnology) as a primary antibody and horseradish peroxidase (HRP) conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology) as a secondary antibody. The membrane was detected by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz Biotechnology) using BioMax film.

3.2.9 Cell cycling studies

Using NIH3T3 cells, 1×10^6 cells were plated on 35 cm^2 plates or 5×10^4 cells were plated on 6-well plates with regular medium [(Dulbecco's modified Eagle medium (DMEM) + 10% calf bovine serum (CBS) + 1% penicillin-streptomycin (P/S)]. After two days, when cells reach 80% confluence, cells were changed into 1% serum medium (DMEM + 1% BCS + 1% P/S) for 1 day. At t=0, cells were replaced with serum-rich medium (50% horse serum + DMEM + 1%

P/S, Balsalobre *et al*., 1998) for two hours. After that, cells were changed into serum-free medium (DMEM + 1% P/S). At each indicated time point, cells were washed by PBS, trypsinized, collected by centrifugation and frozen at -80°C for protein analyses or Trizol (Invitrogen) applied directly onto the cells, collected and frozen at -80°C for RNA analyses.

RNAs was extracted according to the manufacturer's instruction (miRNA kit, Qiagen) and treated by RNase-free DNase I (Promega) to remove genomic DNA. 1 μg of RNA sample from each time point was reverse transcribed by SuperScript® III reverse transcriptase (Invitrogen) into cDNA using hexamer oligonucleotides and oligo dT primers in a 20 μl reaction. Expression of *Dbp, Nr1d1,* and *Nrf1* genes using the respective primer sets (**Table 3.4**) was analyzed by quantitative real-time PCR (QPCR) using an Applied Biosystems 7300 Real Time PCR system. SYBR green (Power SYBR® Green PCR Master Mix, Applied Biosystems) was used as a fluorescent dye and each cDNA sample was loaded in triplicate. For each sample, a ΔC_T value was obtained by normalizing each gene of interest against *GAPDH* in each assay. The $\Delta\Delta C_T$ value was obtained by subtracting the ΔC_T values for each sample from the ΔC_T values of *GAPDH* in each corresponding assay to calculate a relative $\Delta\Delta C_T$. All $\Delta\Delta C_T$ values were converted to fold change.

For protein analysis, nuclear extracts were obtained from each time point (Nuclear Extract Kit, Active Motif) and protein concentration was measured. Samples were loaded onto a Criterion XT 4-12% Bis-Tris Gel (Bio-Rad) for protein separation, followed by transfer to a western blot and subsequent detection using anti-rabbit NRF1 (received from Dr. Daniel Reines), anti-rabbit DBP (Santa Cruz Biotechnology), or anti-rabbit β-actin (ab8227, Abcam).

Table 3.4. RT-PCR primers for gene expression studies (mouse NIH3T3 cells).

Target gene (primer)[a]	Primer number	Exon	Primer sequences
Clock (F)	RN3770	7	5′-CTTAGTAATGAAGAGTTTACACAG-3′
Clock (R)	RN3771	8	5′-TAACGAAGTTACACTCTCAGATAC-3′
Per1 (F)	RN3772	16-17	5′-CCAAGAAAGATCCGTCGTCAGC-3′
Per1 (R)	RN3773	17	5′-AGGGCGAGCGGGCTCAGGGT-3′
Cry1 (F)	RN3774	8	5′-CTGTGGGTTTTGGTAGGAGGAC-3′
Cry1 (R)	RN3524	9	5′-ATTTTGCAGGGAAGCCTCTTAG-3′
Nr1d1 (F)	RN3525	6	5′-CTTCCGTGACCTTTCTCAGC-3′
Nr1d11 (R)	RN3526	7	5′-GCAAAGCGCACCATCAGCAC-3′
Dbp (F)	RN3775	3-4	5′-CTGAGGAACAGAAGGATGAGAAG-3′
Dbp (R)	RN3776	4	5′-ATCTGGTTCTCCTTGAGTCTTC-3′
Nrf1 (F)	RN3807	9	5′-ATTACTCTGCTGTGGCTGATGG-3′
Nrf1 (R)	RN3808	10	5′-CGTCGTCTGGATGGTCATTTC-3′
Nrf1 3’-UTR (F)	RN3945	12	5'-GCCCGTGTTCCTTTGTGGTG-3'
Nrf1 3’-UTR (R)	RN3946	12	5'-GGAGAACAACACAGATTCCATGC-3′
Arntl (F)	RN3779	12-13	5′-TACCTGTTCAAAGAAAAAAGCAG-3′
Arntl (R)	RN3780	13	5′-GGCTCATTGTCTTCGTCCAGC-3′
Per2 (F)	RN3777	10	5′-CCTCCTGGGCTATCTACCTC-3′
Per2 (R)	RN3778	11	5′-CGGTGGACAGCCTTTCGATTA-3′
Clock alternative splicing (F)	RN4066	15	5′-GCGAGAACTTGGCATTGAAGAG-3′
Clock alternative splicing (R)	RN4067	21	5′-CTGTGTCCACTCATTACACTCTGTTG-3′

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer; ex: exon; in: intron. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Mus_musculus/Info/Index).

3.3 RESULTS

3.3.1 Putative NRF1 sites in 5' regulatory elements of ~56% circadian regulatory genes

To determine whether NRF1 may have a role in regulating circadian genes, we first identified 25 potential NRF1 target genes (**Table 3.5**) from bioinformatics searches of 45 candidate genes (**Table 3.5 & Table 3.6**) in human and mouse genome sequences, with strong matches to the NRF1 canonical binding site, 5'-YGCGCANGCGCR-3'. This sequence is a pyrimidine (Y)-purine (R) repeating motif, with both a direct repeat and a palindrome (in each case, 5'-YGCGCR-3'); either of the latter two features is likely responsible for NRF1 binding DNA as a homodimer (Gugneja & Scarpulla, 1997; Gomez-Cuadrado *et al*., 1995). Prior biochemical footprinting studies have shown that NRF1 contacts most or all guanines on both strands of DNA (Virbasius & Scarpulla, 1994), and extensive phylogenetics, *in vitro* and *in vivo* studies in our laboratory and others (Rodriguez-Jato *et al*., 2005, unpublished data) are consistent with the essential nature of the two GCGC sub-motifs. One nucleotide substitution in one GCGC motif is allowed, but reduces affinity, while two substitutions prevent NRF1 binding. Only the Y, N, and R positions are variable, with consensus sequences at these positions increasing affinity. Second, for candidate NRF1 target circadian genes in human and mouse (**Table 3.5**), we assessed phylogenetic conservation by multi-sequence alignment for which the majority of identified TF sites were present in all or most sequenced mammalian genomes (**Figure 3.1 & Appendix A**), indicating functional importance.

(a) Multi-sequence alignment of *CLOCK* promoter

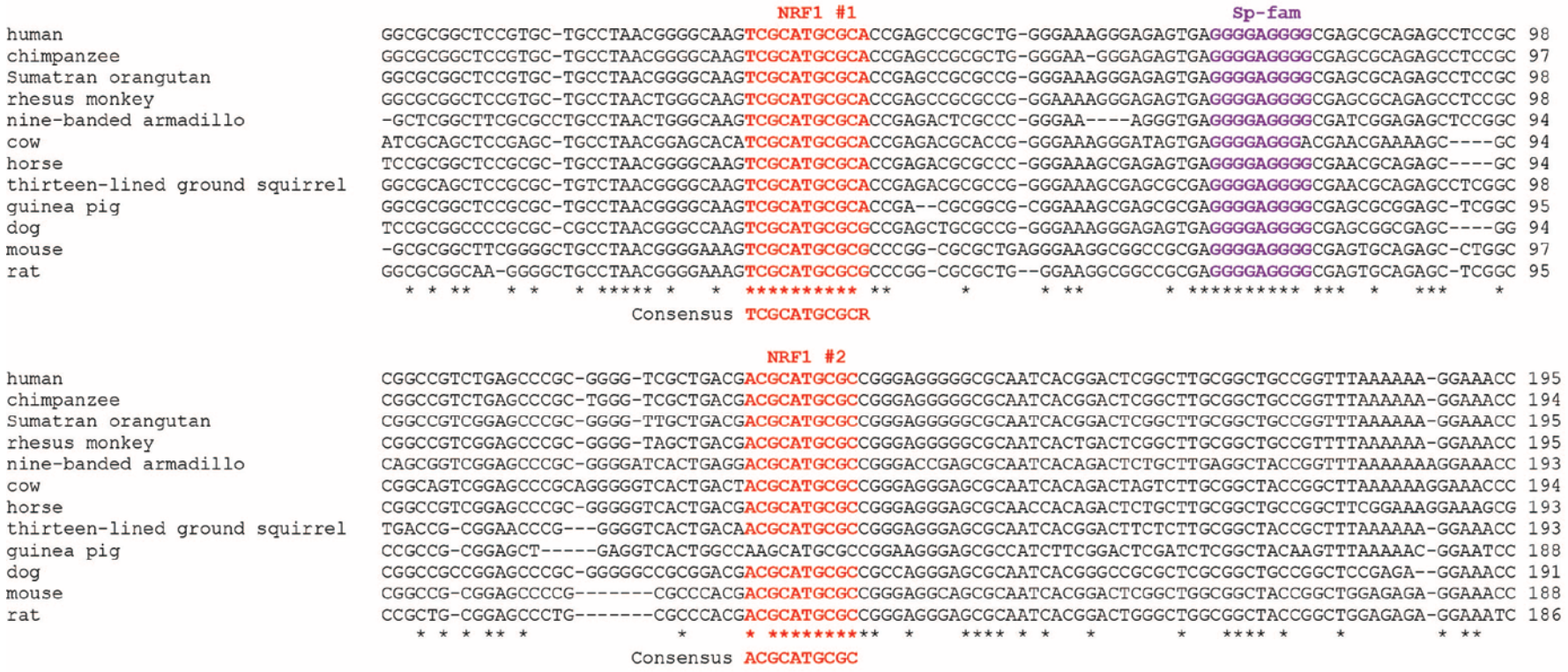


Figure 3.1. (a) (continued below)

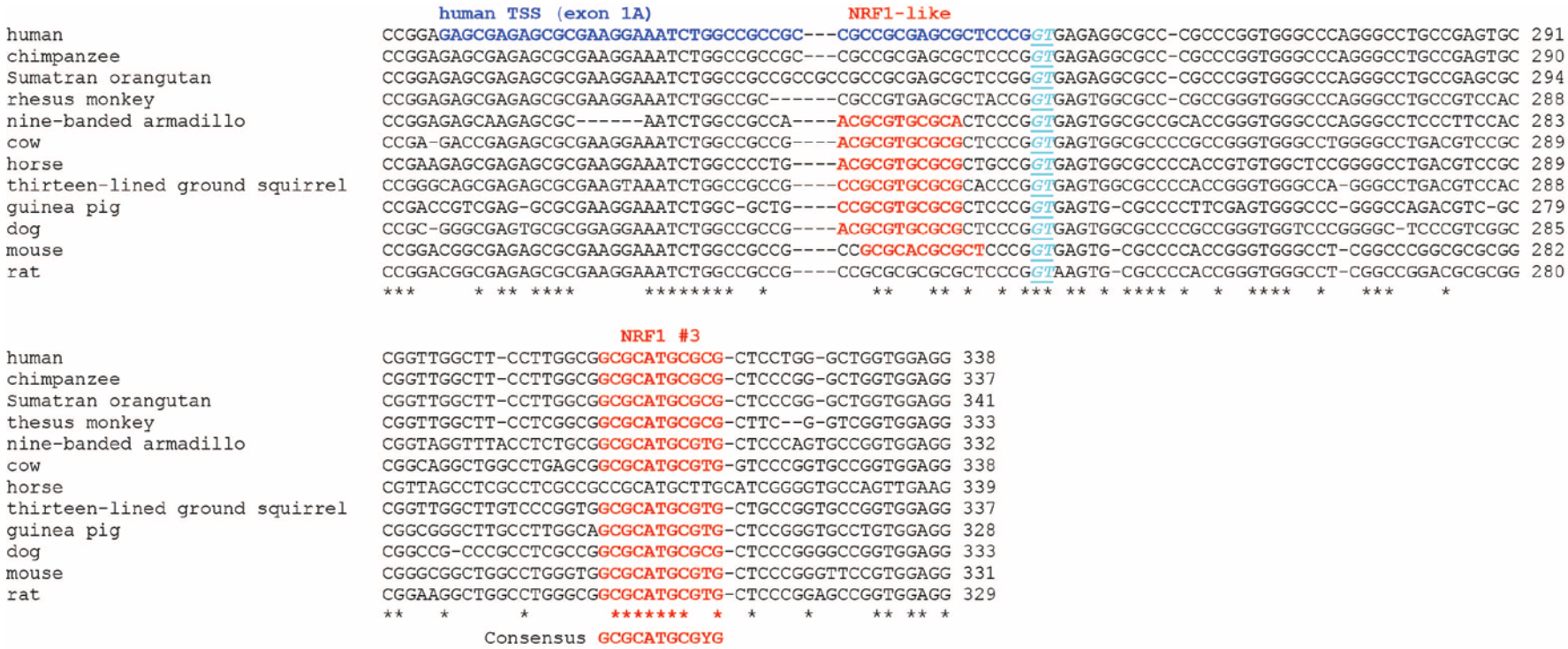


Figure 3.1. (a) (continued below)

(b) Multi-sequence alignment of *CRY1* promoter

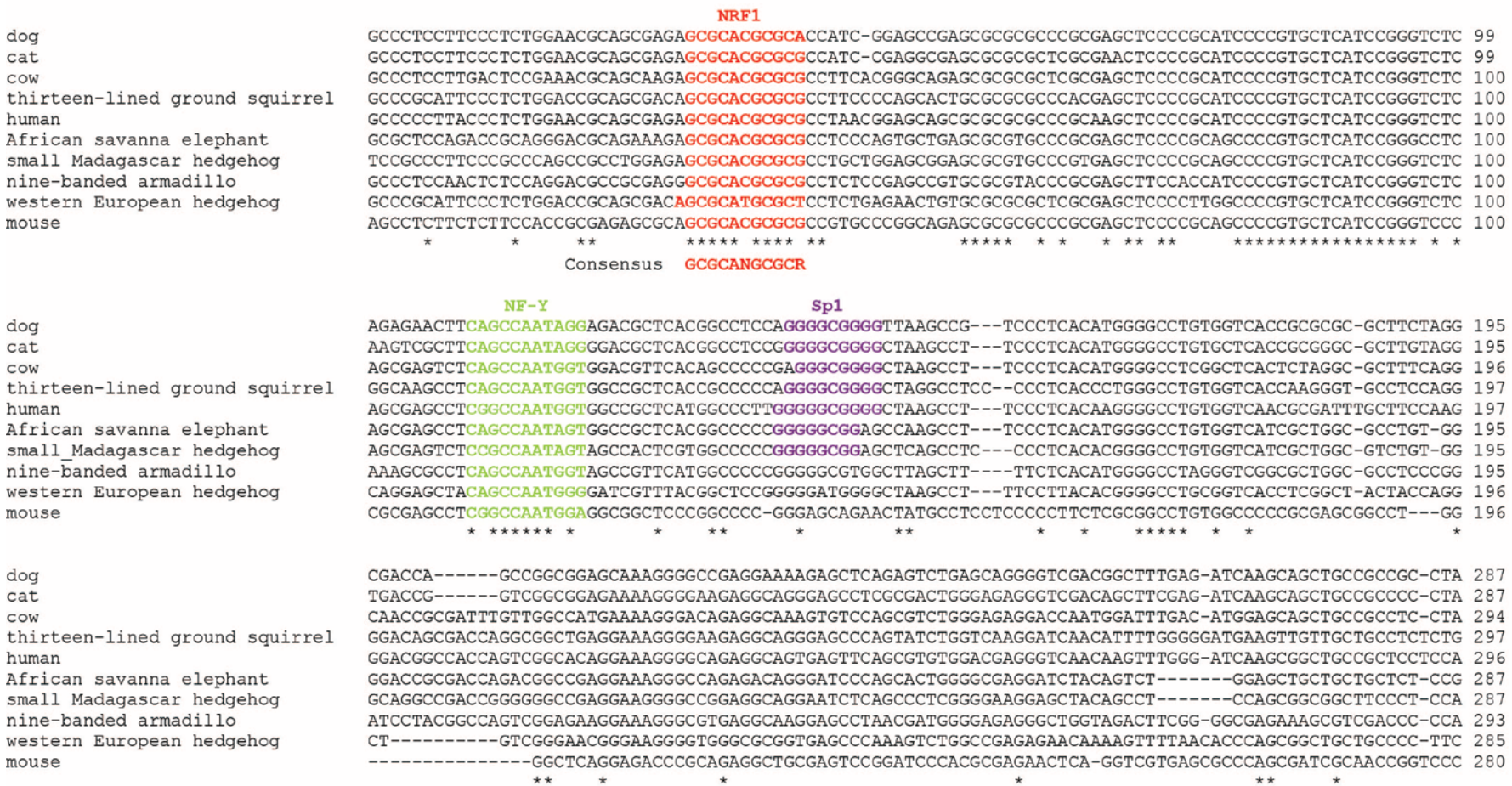


Figure 3.1. (b) (continued below)

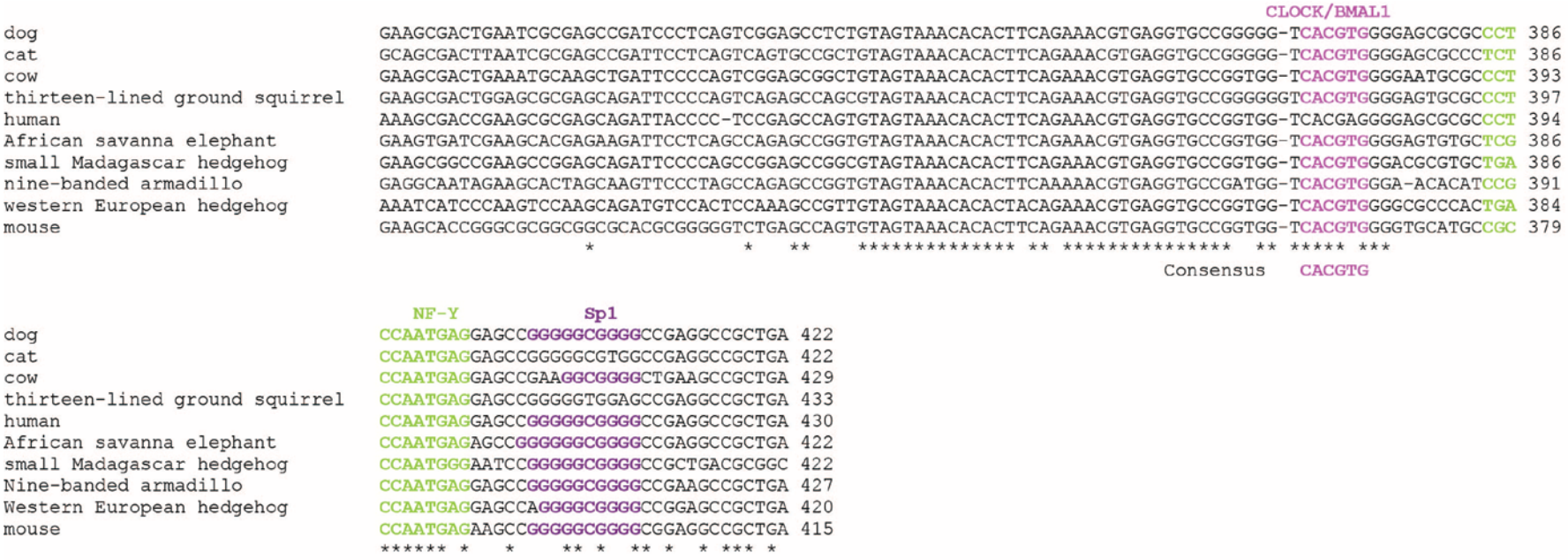


Figure 3.1. (b) (continued below)

(c) Multi-sequence alignment of *PER1* promoter

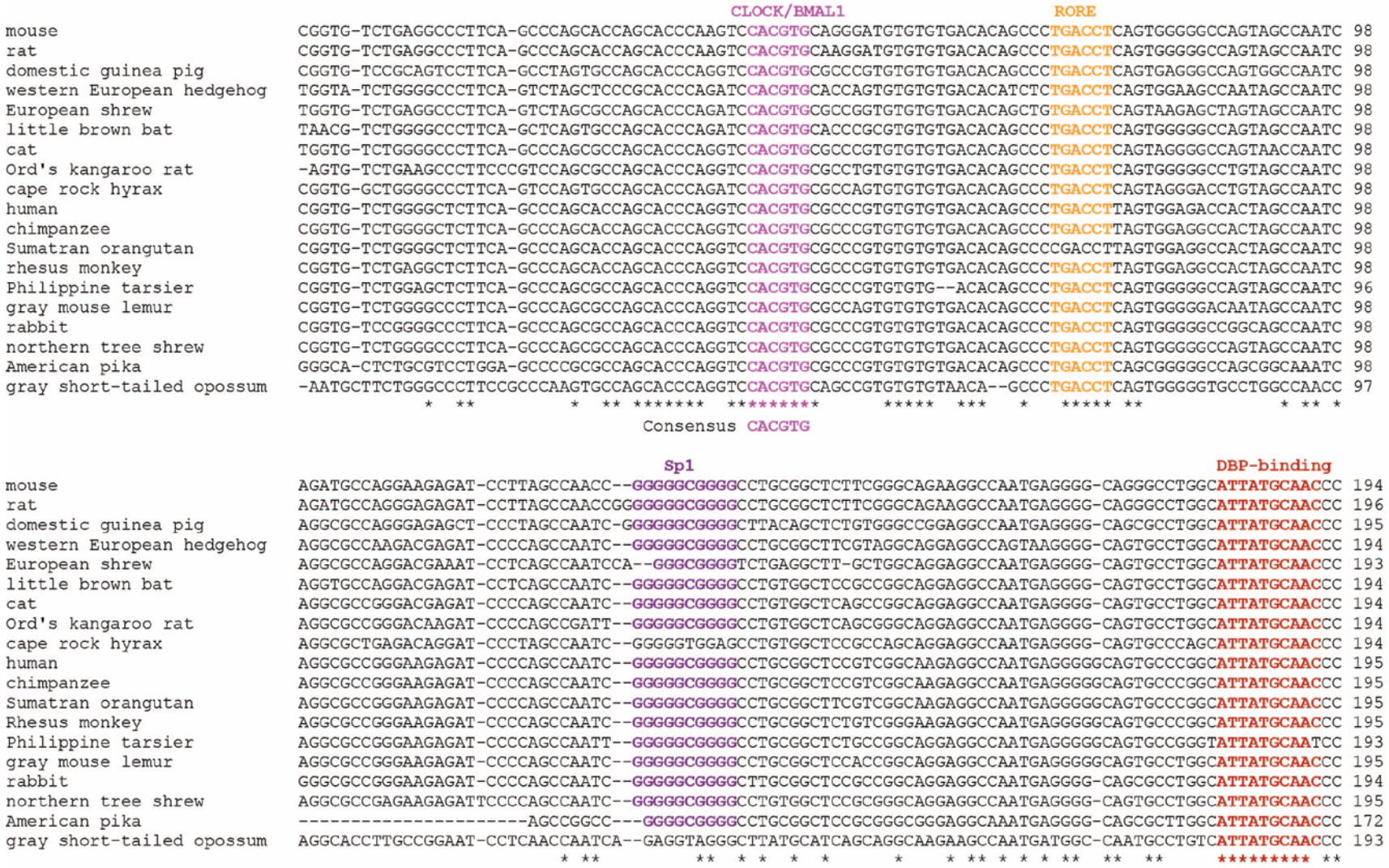


Figure 3.1. (c) (continued below)

(d) Multi-sequence alignment of *PER1* intron 1

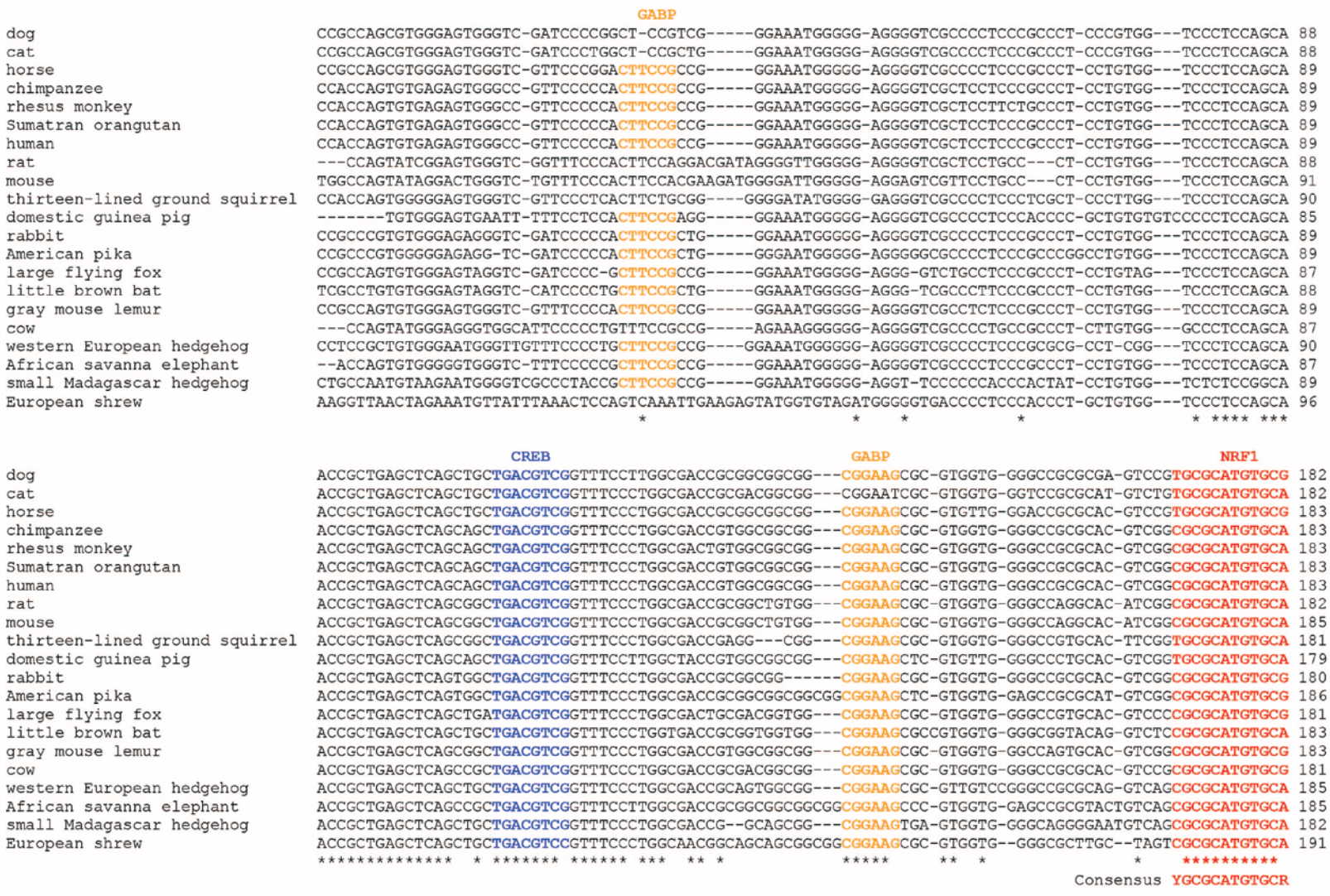


Figure 3.1. (d) (continued below)

Figure 3.1. (d) (continued below)

(e) Multi-sequence alignment of *NR1D1* promoter

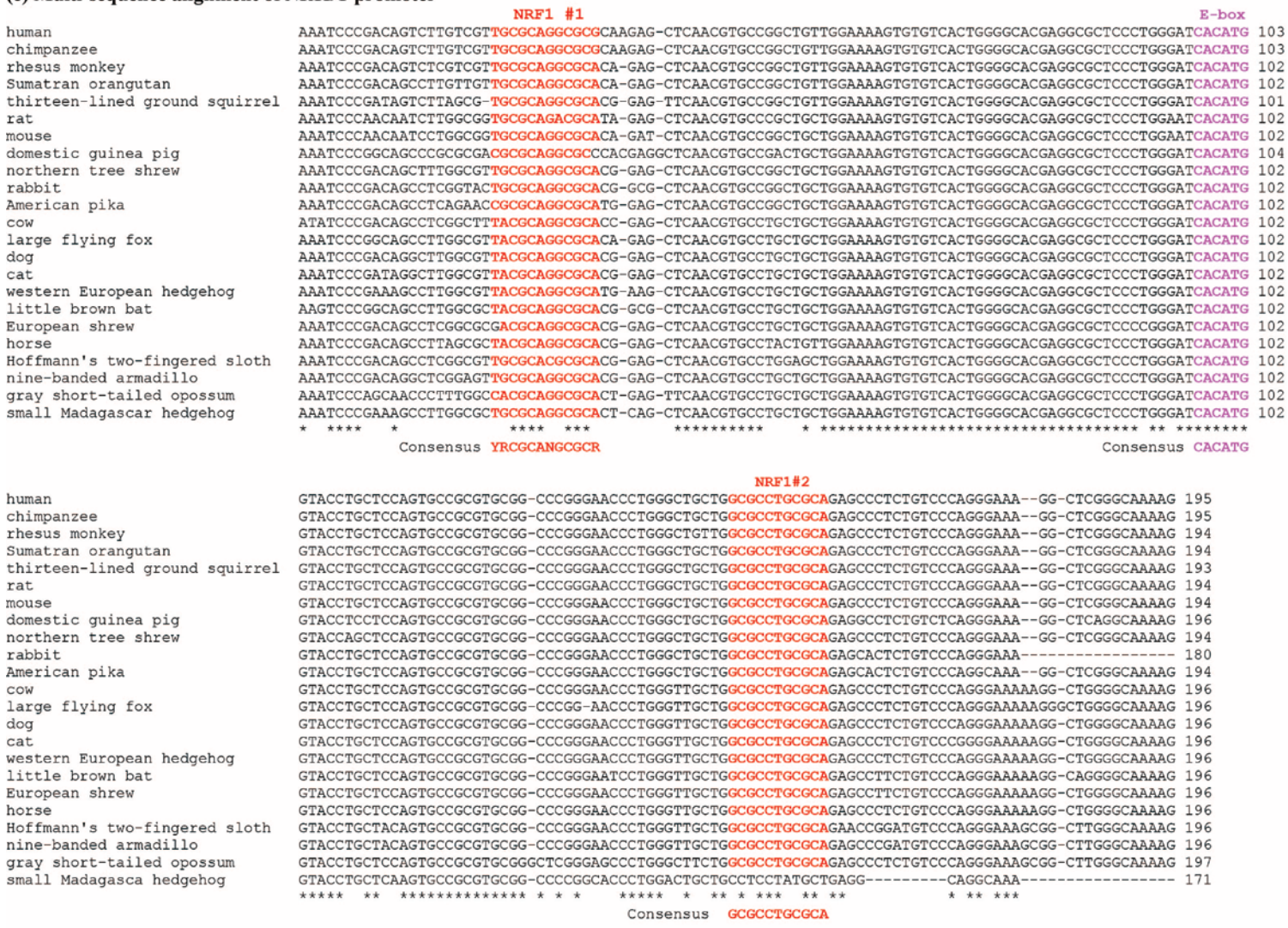


Figure 3.1. (e) (continued below)

(f) Multi-sequence alignment of *DBP* promoter

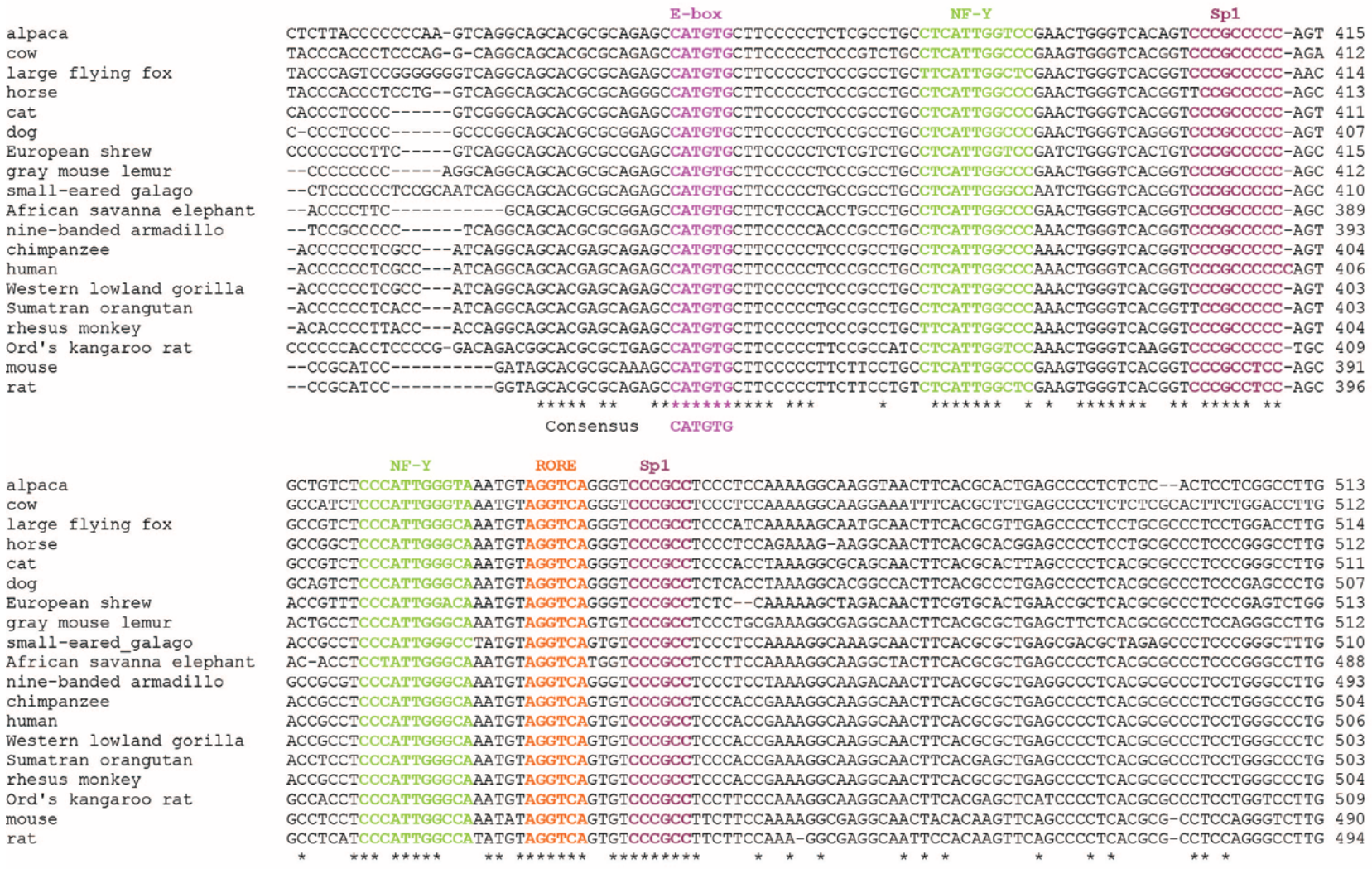


Figure 3.1. (f) (continued below)

Figure 3.1. (f) (continued below)

(g) Multi-sequence alignment of *DBP* intron 2 enhancer

Figure 3.1. Multi-sequence alignments for (a) *CLOCK* promoter; (b) *CRY1* promoter; (c) *PER1* promoter; (d) *PER1* intron 1; (e) *NR1D1* promoter; (f) *DBP* promoter; and (g) *DBP* intron 2 enhancer. Consensus sequences for CLOCK/BMAL1 (E-box) and NRF1 motifs are highlighted in pink and red, respectively.

Table 3.5. NRF1 targets include 25 genes encoding core, output, or input circadian regulators.

Gene	NRF1 site	Human chromosome	Circadian regulatory function	Circadian reference
CLOCK	Promoter, Intron 1	4q12	bHLH TF; forms heterodimer with BMAL1; binds to E-box to activate gene expression; part of positive limb of circadian rhythms; possesses histone acetyltransferase activity	Steeves *et al*., 1999; Hirayama *et al*., 2007; Kondratov *et al*., 2003
PER1	Intron 1	17p13.1	Negative regulator of circadian rhythms; heterodimerizes with CRY1; *PER1* expression is activated by CLOCK/BMAL1 via binding to a promoter E-box element	Motzkus *et al*., 2007
CRY1	Promoter	12q23.3	A photoreceptor; as of *PER1*	Griffin *et al*., 1999; Etchegaray *et al*., 2003; Ueda *et al*., 2005; Langmesser *et al*., 2008
NR1D1[b]	Promoter	17q21.1	Encodes an orphan nuclear receptor; activated by CLOCK and BMAL1 and repressed by PER and CRY; a heme sensor that coordinates metabolic and circadian pathways	Preitner *et al*., 2002; Yin *et al*., 2007
DBP	Intron 2	19q13.33	A member of PAR bZIP TFs; *DBP* gene expression regulated by CLOCK/BMAL1 complex	Ripperger & Schibler U, 2006; Stratmann *et al*., 2010
BTRC[a]	Promoter	10q24.32	Part of the E3 ubiquitin ligase complex, mediates PER1 and PER2 degradation	Shirogane *et al*., 2005; Reischl *et al*., 2007;
PPP5C	Promoter	19q13.3	Part of an E3 ubiquitin ligase complex, mediates PER1 and PER2 degradation	Partch *et al*., 2006

Table 3.5. (continued)

Gene	NRF1 site	Human chromosome	Circadian regulatory function	Circadian reference
FBXL15	Promoter	10q24.32	Protein phosphatase 5; activates the kinase activity of CKIε (encoded by *CSNK1E*) by preventing autophosphorylation	Koh *et al*., 2006
WDR5	Promoter	9q34.2	A component of the histone methyltransferase complex; involved in H3K4 methylation; associates with PER1	Brown *et al*., 2005; Schuetz *et al*., 2006;
TIMELESS	Intron 1	12q13.3	Interacts with CLOCK in circadian rhythms; interacts with Chk1 and the ATR-ATRIP complex in cell cycle checkpoints	Unsal-Kacmaz *et al*., 2005
TEF	Promoter	22q13.2	PAR bZip protein; circadian output gene and TF similar to DBP	Fonjallaz *et al*., 1996
HSF1	Promoter	8q24.3	Activates expression of heat shock proteins by thermal stress; maintains physiological homeostasis of circadian rhythms	Reinke *et al*., 2008; Kim *et al*., 2005
EZH2	Promoter	7q36.1	A polycomb group enzyme; a component of CLOCK/BMAL1 complex; can bind to di- or trimethylation of H3K27 on *Per1* and *Per2* promoters; enhances mCRY-mediated transcriptional repression	Etchegaray *et al*., 2006;
CIPC	Promoter	14p24.3	Additional negative-feedback regulator to CLOCK/BMAL1	Zhao *et al*., 2007
CHORDC1	Promoter	11q14.3	A potential transcription regulator during development and has circadian expression pattern in mammalian brain	Gerstner & Landry, 2007
ALAS1	Promoter	3p21.2	Regulated by CLOCK; rate limiting enzyme in heme biosynthesis	Kaasik & Lee, 2004;

Table 3.5. (continued)

Gene	NRF1 site	Human chromosome	Circadian regulatory function	Circadian reference
FMR1	Promoter	Xq27.3	homolog *dfmr1* (*Drosophila*) mutant shows erratic locomotor activity; Fmr1 KO mice have a shorter running period in 12 hr dark-12 hr dark cycles	Dockendorff *et al*., 2002; Inoue *et al*., 2002; Zhang *et al*., 2008;
FXR2	Promoter	17p13.1	Autosomal homolog of FMR1, similar function as *FMR1*	Zhang *et al*., 2008
CSNK1E	Intron 1	22q13.1	A member of the casein kinase I (CKI) gene family; phosphorylates PER, CRY, and BMAL1	Eide *et al*., 2002; Etchegaray *et al*., 2009
NR1D2[b]	Intron 1	3p24.2	Belongs to REV-ERB family, as for *NR1D1*, uses heme as a ligand	Raghuram *et al*., 2007; Liu *et al*., 2008;
CREM	Promoter	10p11.21	a bZIP TF that binds to the cAMP responsive element, encodes the transcriptional repressor ICER, whose levels in SCN is regulated by light and expressed at high level during the night	Stehle *et al*., 1993; Foulkes, *et al*., 1996;
MYBBP1A	Promoter	17p13.3	Interacts with mCRY1, co-repressor of *PER2* promoter	Hara *et al*., 2009
RAB3A	Intron 1	19p13.2	A ras-associated binding protein; mutation of mouse gene shows abnormal circadian period	Kapfhamer *et al*., 2002
ATF5	Intron 1	19q13.3	Activating transcription factor 5; regulated by CLOCK/BMAL1	Lemos *et al*., 2007
PROK2	Promoter	3p13	A circadian output gene expressed in the SCN; predominantly entrained by endogenous clock, and light is a modulator for its rhythmic expression	Cheng *et al*., 2002; Cheng *et al*., 2005

[a] *NR1D1* is also known as *REV-ERBα*, *NR1D2* as *REV-ERBβ*, and *BTRC* as ß-TrCP1.

3.3.2 NRF1 binds to 5' regulatory elements of a subset of genes involved in circadian regulation and regulates their gene expression

To test if the predicted NRF1 targets are functional, ChIP assay was used to examine *in vivo* binding of NRF1 in human SK-N-SH neuroblastoma cells. Using antibody against NRF1 in ChIP, we showed that NRF1 bound to all predicted targets for 16 tested genes, including *CLOCK*, *PER1*, *CRY1*, *DBP* and *NR1D1* (**Figure 3.2**).

Similarly, to test the transcriptional regulatory role of NRF1, we used a pSUPER siRNA plasmid (Brummelkamp *et al*., 2002; Smith *et al*., 2004) that expresses a shRNA targeting *NRF1* exon 2 to knockdown *NRF1* mRNA levels. By knockdown of *NRF1* mRNA to ~50% levels, we found that all predicted target genes have reduced mRNA levels, including *CLOCK*, *PER1*, *CRY1*, *DBP,* and *NR1D1*, but not those for the *GAPDH* control (**Figure 3.3**). Surprisingly, we observed significant knockdown of *BMAL1* mRNA levels (**Figure 3.3**). *BMAL1* is not predicted to be a direct target of NRF1 based on the lack of a canonical NRF1 site from ~50 kb upstream of the *BMAL1* TSS, all intronic sequence, through ~50 kb downstream of the 3'-UTR. This observation indicates the possibility of a secondary effect on *BMAL1*, although an unknown mechanism by which NRF1 can regulate the circadian system cannot be excluded.

Table 3.6. 21 genes encoding core, output, or input circadian regulators that do not have promoter or intragenic canonical NRF1 sites.

Gene[a]	NRF1 site	Human chromosome	Circadian regulatory function	Reference
BMAL1	none	11p15.2	Heterodimerizes with CLOCK	Hirayama *et al*., 2007
CRY2	none	11p11.2	Paralog of CRY1	Griffin *et al*., 1999; Langmesser *et al*., 2008
PER2	none	2q37.3	Paralog of PER1	von Schantz *et al*., 2006; Kaasik & Lee, 2004
PER3	none	1p36.23	Paralog of PER1	von Schantz *et al*., 2006
NPAS2	none	2q11.2	CLOCK-related; heterodimerizes with BMAL1	Kaasik & Lee, 2004;
BMAL2	none	12p11.23	Heterodimerizes with CLOCK, paralog of BMAL1	Sasaki *et al*., 2009; Shi *et al*., 2010
DEC1	none	3p26.1	bHLH TF; interacts with ARNTL and suppresses CLOCK/BMAL1 complex	Sato *et al*., 2004; Butler *et al*., 2004; Li *et al*., 2003; Honma *et al*., 2002
DEC2	none	12p12.1	bHLH TF; interacts with BMAL1 and suppresses CLOCK/BMAL1 complex; expression is mediated by DEC1	Noshiro *et al*., 2004; Butler *et al*., 2004
FBXL3	none	13q22.3	Forms SCF ubiquitin ligase complexes with Skp1 and cullin proteins to ubiquitinate and degrade CRY1 and CRY2	Busino *et al*., 2007;
NONO	none	Xq13.1	Associates with PER; acts as a bridge between the CREB/TORC complex and RNA polymerase II	Brown *et al*., 2005; Amelio *et al*., 2007;
PPARGC1A	none	4p15.2	A transcriptional coactivator regulates genes involved in energy metabolism; stimulates BMAL1, NR1D1, NRF1 and NRF2 (GABP)	Liu *et al*., 2007

Table 3.6. (continued)

Gene[a]	NRF1 site	Human chromosome	Circadian regulatory function	Reference
RORA	none	15q22.2	A retinoid-related orphan receptor; activates BMAL1 transcriptional activity through RORA binding box	Akashi & Takumi, 2005
RORC	none	1q21	Also a retinoid-related orphan receptor; mostly expressed in skeletal muscle	Hirose *et al*., 1994
CSNK1D	none	17q25	A member of the casein kinase I (CKI) gene family; maintains 24 hr circadian cycle length by phosphorylation regulation of PER proteins	Etchegaray *et al*., 2009
AANAT	none	17q25	An acetyltransferase; a penultimate enzyme in melatonin synthesis, regulated by cAMP-dependent phosphorylation	Hintermann *et al*., 1996; Coon *et al*., 2001
HLF	none	17q22	A member of the PAR TF family; a clock output gene	Young *et al*., 2001; Young *et al*., 2002
CCRN4L	none	4q31.1	Also known as Nocturnin in mouse; a deadenylase in circadian output pathways	Garbarino-Pico *et al*., 2007; Green *et al*., 2007
E4BP4	none	9q22	Phosphorylation regulation by *CSNK1D*; negatively regulates expression of *Per2*	Doi *et al*., 2004; Ohno *et al*., 2007
CSNK2A1	none	20p13	A subunit of casein kinase II (CK2); CK2 phosphorylates PER2 and regulates PER2 stability with CSNK1E	Tsuchiya *et al*., 2009
CSNK2A2	none	16q21	Also a subunit of casein kinase II, as for CSNK2A1	Tsuchiya *et al*., 2009
CSNK2B	none	6p21.3	Also a subunit of casein kinase II, as for CSNK2A1	Akten *et al*., 2003; Tsuchiya *et al*., 2009

[a] *BMAL1* is also known as *ARNTL*, *BMAL2* as *ARNTL2*, *DEC1* as *BHLHB2*, *DEC2* as *BHLHB3*, *PPARGC1A* as PGC-1α, *CCRN4L* as *NOCTURNIN*, *E4BP4* as *NFIL3*, *CSNK2A1* as CSK2alpha, *CSNK2A2* as CSK2alpha', and *CSNK2B* as CSK2beta.

Figure 3.2. NRF1 ChIP assays using SK-N-SH neuroblastoma cells. *SNURF-SNRPN* enhancer is a positive control. Abbreviations: Ab+: antibody positive; Ab-: antibody negative; TI: total input of DNA; H_2O: PCR negative control; bp: base pair.

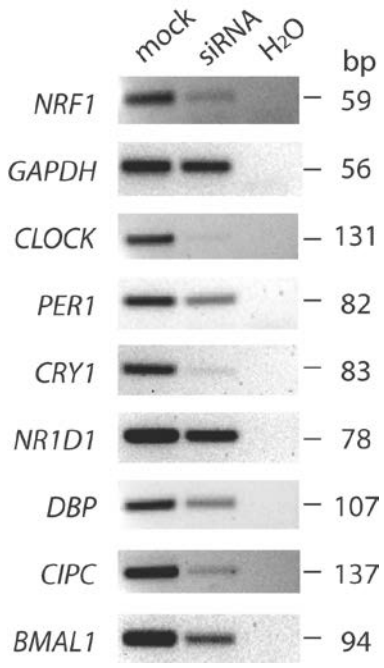


Figure 3.3. *NRF1* siRNA assays using SK-N-SH neuroblastoma cells. *GAPDH* is a negative control. Mock: no siRNA transfection negative control. siRNA: *NRF1* siRNA transfection; H_2O: PCR negative control; bp: base pair.

3.3.3 NRF1 regulates expression through the promoters of *CLOCK*, *CRY1* and *NR1D1*, and the enhancers of *PER1* and *DBP*

To test the function of NRF1 sites in the 5' regulatory regions of major circadian regulatory genes, luciferase reporter constructs for *CLOCK*, *PER1*, *CRY1*, *NR1D1* and *DBP* were made using the conserved promoter sequences of *CLOCK*, *DBP*, *CRY1*, *PER1*, and *NR1D1*, and the conserved enhancer sequences of *DBP* and *PER1* [**Figure 3.4(a), Figure 3.1**].

For the *CLOCK* promoter, two luciferase constructs were made in pGL3basic [**Figure 3.4(b)**], one with 2 intact NRF1 sites (CLOCK-pr) and the other with a site-specific mutation in the second NRF1 site [CLOCK-pr-mu, **Figure 3.4(a)**]. As expected, mutation of the second NRF1 site in the promoter significantly reduces the luciferase activity compared to the intact promoter construct in transfected SK-N-SH neuroblastoma cells [**Figure 3.4(b)**].

For the *CRY1* promoter, three constructs have been made [**Figure 3.4(c)**] with variation in the size of the promoter. Compared to the full-length *CRY1* promoter (CRY1-pr), the luciferase construct CRY1-pr-s2 with a deletion of just the NRF1 site has significantly reduced the luciferase activity (by ~30%, $P<0.01$), indicating that NRF1 is a transcription activator of the *CRY1* promoter. To examine the effects on transcriptional activity of loss of E-box motif (Ueda *et al*., 2005), as well as NF-Y and Sp1 sites, in the *CRY1* promoter, we also made a short version of *CRY1* promoter with only the NRF1 and NF-Y site [CRY1-pr-s1, **Figure 3.4(c)**]. Surprisingly, this construct showed a much higher activity than the full-length of *CRY1* promoter construct. Two potential mechanisms can explain this observation. First, either or both of two, long conserved blocks of sequence 5' to the E-box [**Figure 3.1(b)**] could bind a repressor of *CRY1* transcription, which would be removed in the CRY1-pr-s1 construct. Alternatively, the increased activity of this construct versus the full-length construct may be due to shortening the distance from the NRF1 and NF-Y sites to the TSS. Indeed, the most common position of the NRF1 motif in genome-wide bioinformatics analyses is centered at 62-nt 5' of the TSS (Xie *et al*., 2005), so the NRF1 site in the CRY1-pr-s1 construct is likely to be in a more optimal position compared to the position in the wildtype *CRY1* promoter construct.

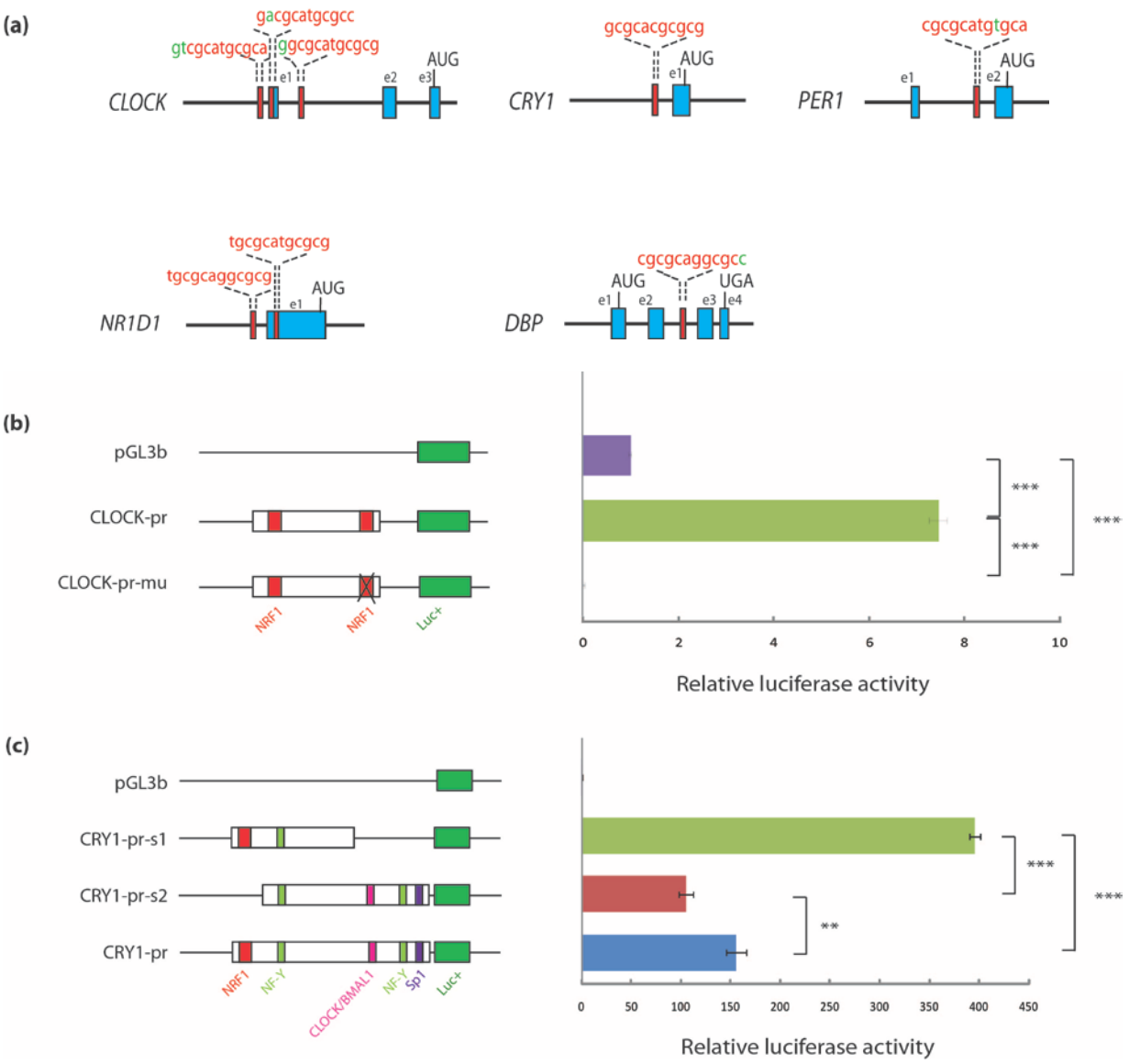


Figure 3.4. (continued below)

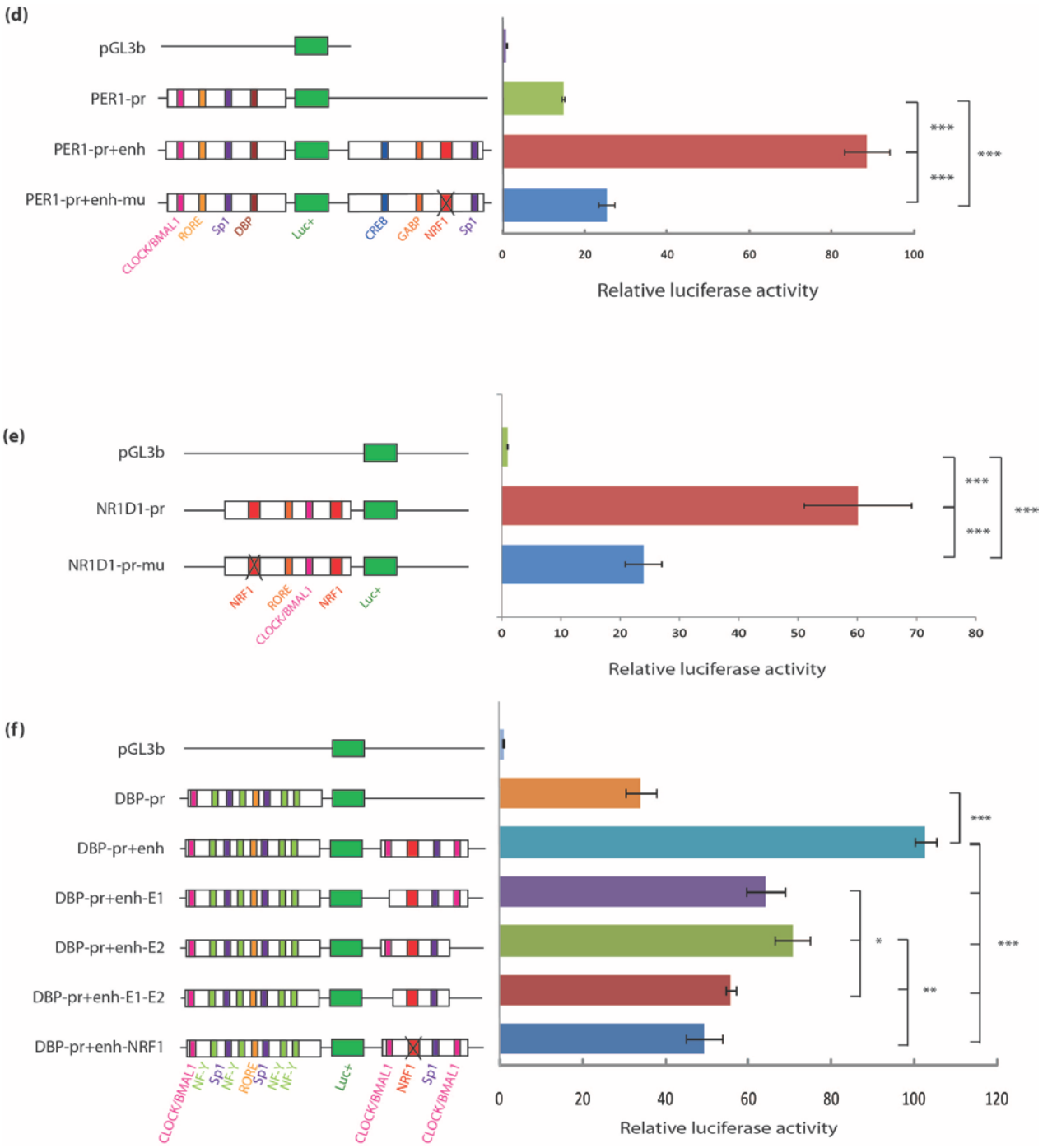


Figure 3.4. Luciferase reporter assays of 5' regulatory elements of *CLOCK*, *CRY1*, *PER1*, *NR1D1* and *DBP*. (a) Gene structure for *CLOCK*, *CRY1*, *PER1*, *NR1D1* and *DBP*, with sequence and location of NRF1 putative binding sites. For NRF1 sites, red represents nucleotides matching

to the NRF1 consensus recognition site and green represents less optimal nucleotides. Luciferase reporter assays for (b) the *CLOCK* gene promoter in SK-N-SH neuroblastoma cells, (c) the *CRY1* gene promoter in SK-N-SH neuroblastoma cells, (d) the *PER1* gene 5' regulatory element in SK-N-SH neuroblastoma cells, (e) the *NR1D1* gene promoter in NIH3T3 cells and (f) the *DBP* gene 5' regulatory element in NIH3T3 cells. $P<0.05$: *; $P<0.01$: **; $P<0.001$: ***.

Similarly, *PER1* 5' regulatory elements were cloned into the luciferase vectors [**Figure 3.4(d)**]. Compared to the *PER1* promoter construct (PER1-pr), the luciferase activity of a construct (PER1-pr+enh) carring the *PER1* promoter with an intact putative intron 1 enhancer increased by approximately 5.9-fold [$P<0.001$, **Figure 3.4(d)**] in transfected SK-N-SH neuroblastoma cells. This result confirms our hypothesis that the *PER1* intron 1 NRF1 site is within an enhancer element for *PER1* promoter activity. Not surprisingly, a single site-directed mutation of the NRF1 binding site in the *PER1* enhancer (PER1-pr+enh-mu construct) significantly reduced luciferase activity by approximately 70%, compared to the intact promoter and enhancer construct [$P<0.001$, **Figure 3.4(d)**].

For the *NR1D1* promoter, two luciferase constructs were made [**Figure 3.4(e)**], one with two intact NRF1 sites (NR1D1-pr) and the other with a site-specific mutation of the first NRF1 site (NR1D1-mu). As expected, the construct with the NRF1 site mutation has significantly reduced the luciferase activity by approximately 60% compared to the intact promoter construct in transfected NIH3T3 cells [$P<0.001$, **Figure 3.4(e)**].

Previously, two E-box elements in intron 2 of *DBP* were shown to be critical for circadian transcriptional regulation of this gene in the SCN by CLOCK/BMAL1 (Ripperger et al., 2000; Ripperger and Schibler, 2006). Deletion of the two E-box elements and sequence in between in Rat-1 cell line and in a mouse model showed loss of circadian mRNA expression of *DBP* compared to the wildtype and altered histone modification at the chromatin level. By bioinformatics analyses, we found a conserved, putative NRF1 site between the two E-box elements in intron 2 of *DBP* [**Figure 3.4(a), (f), Figure 3.1(g)**]. To test the role of the NRF1 site in this region, we cloned the *DBP* promoter alone (DBP-pr construct) and with the enhancer element (DBP-pr+enh) into a pGL3basic luciferase reporter vector. As expeced, the *DBP* promoter construct is functional and has increased the luciferase activity by 34-fold ($P<0.01$) compared to the pGL3basic vector, while the enhancer construct (DBP-pr+enh) has increased the transcriptional activity another 3-fold to the *DBP* promoter construct [overall 102-fold compared to the pGL3basic, $P<0.001$, **Figure 3.4(f)**]. Deletion of each individual (DBP-pr+enh-E1, DBP-pr+enh-E2) or both E-boxes (DBP-pr+enh-E1-E2) within the enhancer significantly reduced luciferase and hence enhancer activity by 38%, 32%, and 47%, respectively [$P<0.001$, $P<0.001$, $P<0.001$, respectively, **Figure 3.4(f)**]. Interestingly, mutation of the NRF1 binding site with both E-boxes intact (DBP-pr+enh-NRF1) reduced *DBP* enhancer activity to a greater extent [by 52%, $P<0.0001$, **Figure 3.4(f)**] than mutation of either E-box motif and at least as much as mutations of both E-boxes [**Figure 3.4(f)**]. These data provide strong evidence that NRF1 acts as an activator of the *DBP* gene enhancer.

As a complementary approach, we used the intact *DBP* promoter+enhancer luciferase construct (DBP-pr+enh) and co-transfected with expression vectors for NRF1 (NRF1-VP16; Ramachandran *et al*., 2008), CLOCK and BMAL1 (Kondratov *et al*., 2006). CLOCK and

BMAL1 increased luciferase activity of the DBP-pr+enh construct by ~18-fold (P<0.001, **Figure 3.5**). Similarly, NRF1 alone or in combination with CLOCK and BMAL1 increased luciferase activity of the DBP-pr+enh construct by ~14 and ~21 fold, respectively (P<0.001, **Figure 3.5**). These data demonstrate that NRF1 as well as the CLOCK-BMAL1 heterodimer activate the *DBP* intron 2 enhancer.

Taken together, luciferase reporter assays have demonstrated that NRF1 is a major transcriptional activator for *CLOCK*, *CRY1*, and *NR1D1* though promoter target sites. Similarly, NRF1 acts as a major regulator of *PER1* and *DBP* transcription through intronic enhancer sites.

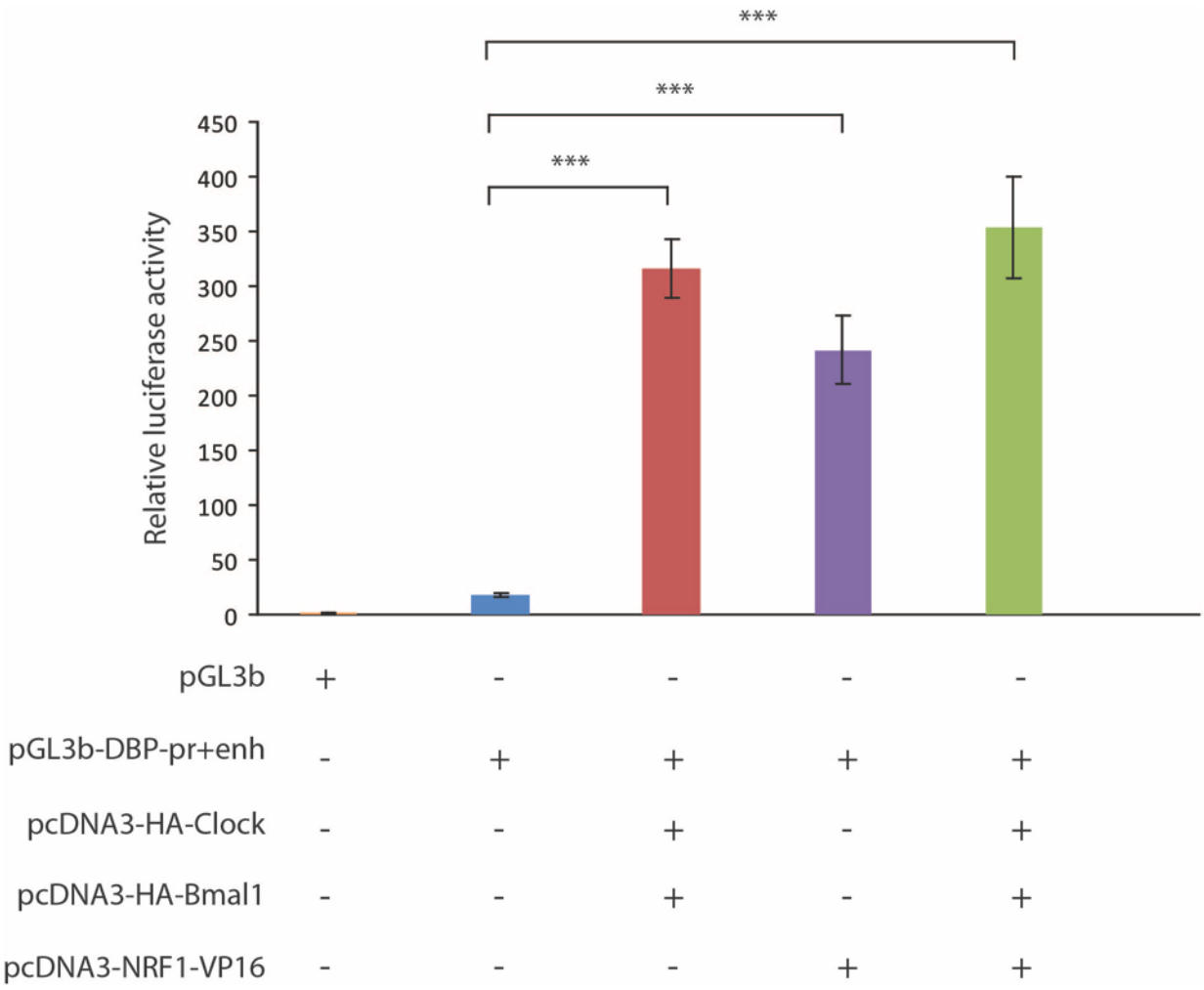


Figure 3.5. Co-transfection of the *DBP* promoter+enhancer luciferase construct with expression vectors for CLOCK, BMAL1 and NRF1. *P*<0.001: ***.

3.3.4 NRF1 and the CLOCK/BMAL1 heterodimer are in a molecular complex

We have shown that NRF1 binds spatially close to two CLOCK/BMAL1 binding sites in the *DBP* intron 2 enhancer and together NRF1 and CLOCK/BMAL1 co-regulate this *DBP* circadian enhancer. Therefore, we hypothesize that NRF1 interacts with CLOCK/BMAL1 in a molecular complex, and that CLOCK may acetylate NRF1 with deacetylation by SIRT1. In support of this hypothesis are the following observations: 1) CLOCK is known to acetylate histone H3, histone H4, and BMAL1 (Doi *et al*, 2006; Hirayama *et al*, 2007); 2) SIRT1 is known to associate with CLOCK/BMAL1 and to promote the deacetylation of histone H3, BMAL1, and PER2 (Asher *et al*, 2008; Nakahata *et al*, 2008) and 3) *in vitro* acetylation of NRF1 by pCAF (p300/CBP-associated factor) enhances its function (Izumi *et al*., 2003), suggesting that NRF1 acetylation *in vivo* may contribute to its mechanism of action at least at a subset of *cis*-regulatory elements that it targets.

The acetylated lysine residue(s) in NRF1 [**Figure 3.6(a)**] are not known (Izumi *et al*, 2003), and other HAT enzymes, including CLOCK, might be relevant for NRF1 acetylation *in vivo*. Few targets of the CLOCK [**Figure 3.6(b)**] HAT enzymatic function have been identified (Doi *et al*., 2006; Hirayama *et al*., 2007). For histone H3, it is lysine 14 (K14) that is acetylated [Doi *et al*, 2006, **Figure 3.6(c)**], while phosphorylation of histone H3 at S10 (which is induced by light, Crosio *et al*, 2000) enhances acetylation at K14 (Doi *et al*., 2006). For BMAL1, it is K537 (mouse) that is acetylated [**Figure 3.6(c)**], whereas the acetylated lysines in histone H4 have not been identified (Doi *et al*., 2006). Our comparison of histone H3 and BMAL1 amino acid sequences around the CLOCK-acetylated lysine identified a shared SXGGK motif with similar motifs present in a region of more extended similarity between histone H4 and NRF1 [SXGRGK;

Figure 3.6(c)]. This observation predicts lysine 221 in NRF1 as a candidate for potential CLOCK acetylation.

To test this hypothesis, co-immunoprecipitation was performed to examine a potential interaction between NRF1 and CLOCK. We used antibodies against NRF1 and, as a positive control, antibodies against BMAL1 for immunoprecipitation and then detected CLOCK proteins on a western blot [**Figure 3.6(d)**]. As expected (Lee *et al*., 2001; Tamaru *et al*., 2003; Kondratov *et al.*, 2006), BMAL1 co-immunoprecipitated both native and phosphorylated CLOCK-isoforms from nuclear extracts. Intriguingly, NRF1 not only co-immunoprecipitated both CLOCK isoforms, but preferentially associated with the phosphorylated, active form of CLOCK [**Figure 3.6(d)**]. Besides full length CLOCK and phosphorylated CLOCK isoforms, we detected a low level of a smaller CLOCK protein in nuclear extract, in NRF1-IP and BMAL1-IP samples [**Figure 3.6(d)**]. According to previous studies, a smaller *Clock* mRNA isoform was found in mouse with alternative splicing of exon 18, which leads to an in-frame 30 amino acid deletion (King *et al*., 1997). Using primers located in exons 15 and 21 (**Table 3.4**), RT-PCR was performed using cDNA from NIH3T3 cells (see below). As expected, we found two RT-PCR bands [**Figure 3.6(e)**], the sizes which match the more abundant full-length and the less abundant alternatively spliced (lacking exon 18) *Clock* mRNA isoforms. Therefore, all three protein bands detected by western analysis are different isoforms of CLOCK.

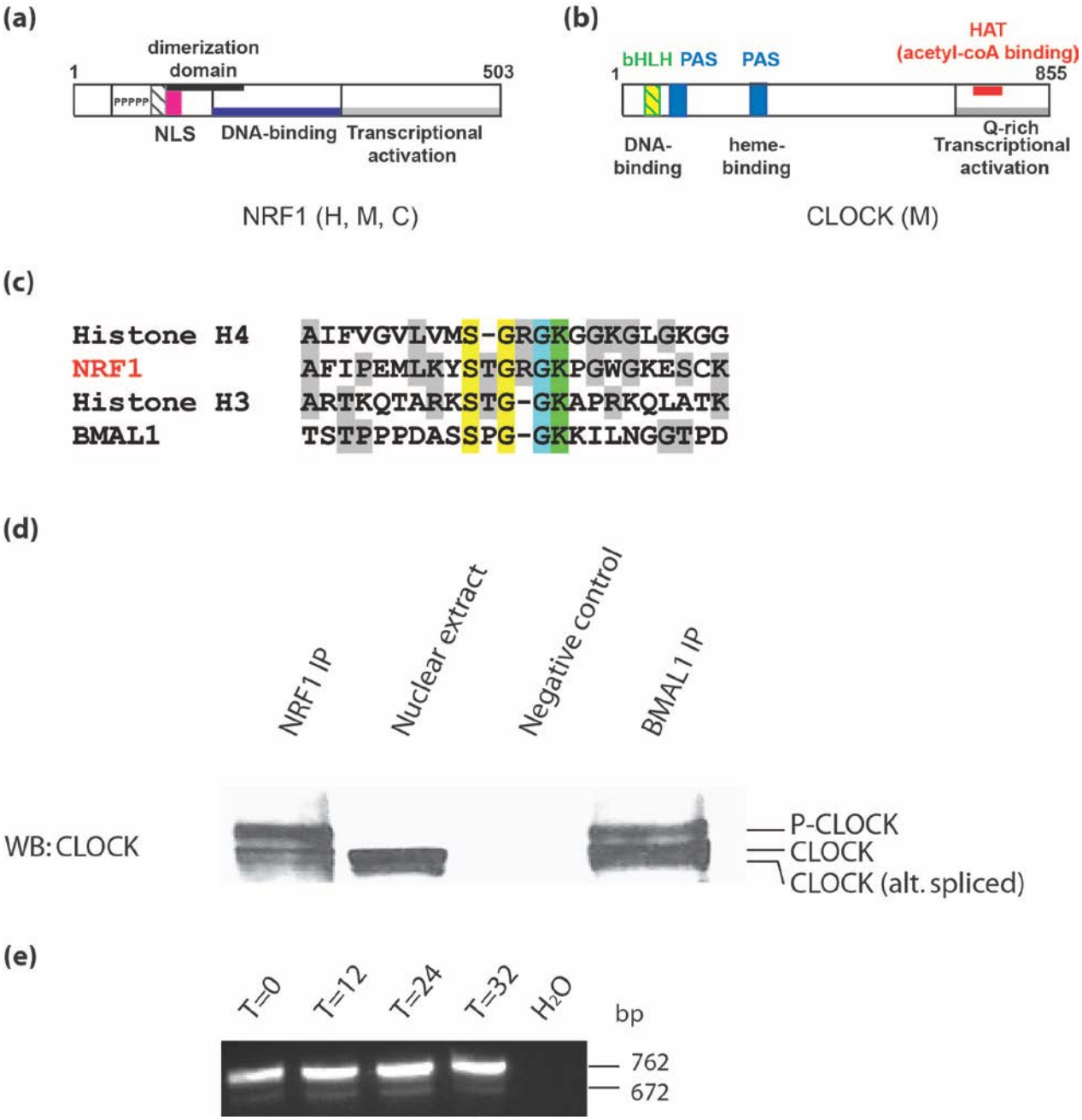


Figure 3.6. Co-immunoprecipition of NRF1 or BMAL1 and CLOCK, as detected by western blot. (a) Structure of the conserved NRF1 polypeptide. NRF1 from human (H), mouse (M), and chicken (C) are 99-100% identical over the entire sequence. The position of structural domains is indicated, including nuclear localization signal (NLS, pink vertical box), DNA binding (dark blue

horizontal box), dimerization (black horizontal bar) an transcriptional activation (grey box) domains. NRF1 has 5 serine residues that are phosphorylated (P). Modified from Scarpulla (2008). **(b)** Structure of the CLOCK polypeptide from mouse (M) showing functional motifs. **(c)** Amino acid alignment of CLOCK-targeted acetylation motifs in histone H3 and BMAL1, and similar motifs in histone H4 and NRF1. The acetylated lysine in H3 and BMAL1 have green shading, with residues present in all 4 sequences in green, blue or yellow shading, while gray shading denotes amino acids present in 2 or 3 of the sequences. **(d)** Detection of CLOCK protein on a western blot, showing native CLOCK, phosphorylated CLOCK (P-CLOCK), and a low level of CLOCK resulting from alternatively spliced (alt. spliced) *Clock* mRNA (see **e**). Abbreviations: IP: immunoprecipitation; nuclear extract: total protein extract from nucleus; negative control: the negative control in immunoprecipitation uses control agarose resin. **(e)** RT-PCR analysis of full-length (762-bp) and alternatively-spliced (672-bp) *Clock* mRNA isoforms. Each lane shows RT-PCR products from NIH3T3 cell cDNA derived from different time points after serum-shock.

3.3.5 Ultradian oscillations of *Nrf1* mRNA and NRF1 protein in serum-shocked NIH3T3 cells

It has been reported that a subset of clock genes can be synchronized and subsequently undergo at least two circadian oscillation cycles in cell line models (Balsalobre *et al*., 1998; 2000; Earnest & Cassone, 2005). To test if *Nrf1* mRNA levels oscillate in a circadian or other fashion, we used serum-shocked NIH3T3 cells, an established model to synchronize circadian cycles of a subset of clock genes (Osland *et al*., 2010). As expected, both *Dbp* and *Nr1d1* showed typical

rhythmic expression in this cell model, with the peak phase at 24 hr and 20 hr, respectively, after serum-shock [**Figure 3.7(a)&(b),** Balsalobre *et al*., 1998], while *Clock* and *Per1* mRNA levels did not cycle (data not shown). These findings are consistent with previous reports with respect to peaks and phases of oscillation and oscillatory behavior of mRNAs encoding known circadian regulators (Balsalobre *et al*., 1998; Osland *et al*., 2010). Interestingly, *Nrf1* mRNA levels oscillated [**Figure 3.7(c)**], with the peak phase at 16 hr and 32 hr, respectively, after serum-shock, indicating an ultradian rhythm (~16 hr; Hughes *et al*., 2009). At the protein level, NRF1 showed a similar ultradian rhythm as for mRNA levels, when compared to the positive control (DBP) and the β-actin negative control [**Figure 3.7(d) & (e)**]. As we have shown that NRF1 controls expression of circadian regulatory genes in both the positive and negative feedback loops, it is not surprising that NRF1 does not cycle in a typical circadian manner. Indeed, even at the nadir of *Nrf1* mRNA and NRF1 protein levels, there are still significant levels present in these cells under these conditions [**Figure 3.7(c),(e)**]. Further studies are needed to determine if NRF1 levels oscillate *in vivo* in the SCN and other tissues.

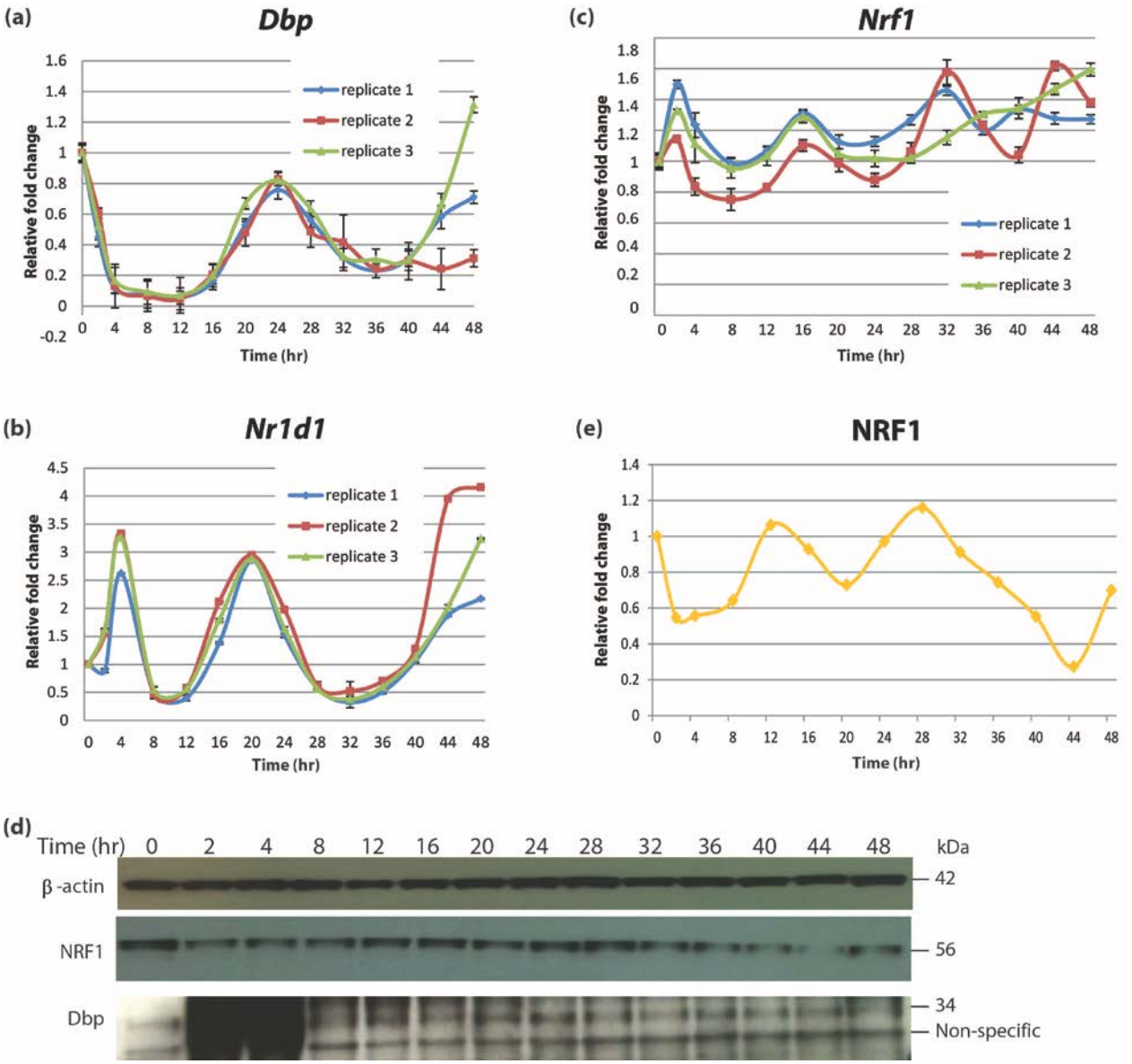


Figure 3.7. NRF1 mRNA and protein expression levels in serum-shocked NIH3T3 cells. (a) mRNA expression levels of *Dbp*. **(b)** mRNA expression levels of *Nr1d1*. **(c)** mRNA expression levels of *Nrf1*. Three different biological replicates are shown. **(d)** Protein expression of DBP and NRF1 detected on western blot. β-actin was used as a control. **(e)** Quantification of protein amounts in each lane for NRF1 normalized to β-actin, using Image J software.

3.4 DISCUSSION

In this study, for the first time NRF1 has been demonstrated to positively regulate numerous circadian genes, including core regulatory genes and CCGs with regulatory roles (**Figure 3.2-3.4, 3.8, Table 3.3**). Overall, ~56% of these genes are targets of NRF1 regulation. Furthermore, NRF1 and the active, phosphorylated CLOCK polypeptide interact in a molecular complex. These observations indicate a significant impact of NRF1 on circadian biology. In particular, we used numerous molecular genetic methods to show that NRF1 positively regulates transcriptional activity of *CLOCK*, *CRY1*, and *NR1D1* through promoter sites, as well as *PER1* and *DBP* through enhancer sites (**Figure 3.8**). Intriguingly, we also observed that *BMAL1*, which was not predicted to be a direct target of NRF1, had down-regulated mRNA levels in *NRF1* siRNA assays. This could be caused by down-regulation of other NRF1 target genes in the circadian system that encode regulators of *BMAL1* expression (**Table 3.3**, **Figure 3.8**) or an unknown circadian factor that is also under NRF1 regulation and remains to be identified. If additional circadian regulatory genes that are not direct targets if NRF1 show a similar secondary effect, then the role of NRF1 in the circadian network would be extremely broad.

NRF1 was first identified with a major role in activating nuclear genes encoding mitochondrial functions and biogenesis (Virbasius *et al*., 1993; Scarpulla, 2008), in cell cycle regulation (Cam *et al*., 2004; Patti *et al*., 2003), neuronal development and function (Chang *et al*., 2005; Yang *et al*., 2006; Liang& Wong-Riley, 2006, Smith *et al*., 2004), in Prader-Willi syndrome (Stefan M, *et al*., in preparation; **Chapter 2**), and is implicated in diabetes (Patti *et al*., 2003; Mootha *et al*., 2003; Gaulton *et al*., 2008; Liu *et al*., 2008; He *et al*., 2008). Studies of NRF1 orthologs have shown that loss of Nrf1 function in zebrafish is embryonic lethal and leads to a specific loss of photoreceptor neurons and their precursors (Becker *et al*., 1998). As circadian

rhythms are entrained by light through retinal afferents to the SCN (Rutter *et al*., 2002), NRF1 could regulate the process of light entrainment and neuronal connections to the SCN, although this hypothesis remains to be examined. Additionally, a serum shock induces circadian gene expression in cell lines (Balsalobre *et al*., 1998), and NRF1 is in an inactive state in serum-starved cells but becomes active (phosphorylated) with serum addition (Gugneja & Scarpulla, 1997). Finally, in the peripheral system, PPARGC1A, a transcriptional coactivator to NRF1 and other TFs in mitochondrial metabolism, is rhythmically expressed in the mouse liver and skeletal muscle, and may play a role in metabolic adaptations to the daily circadian cycle (Liu *et al*., 2007). These observations are consistent with our findings that NRF1 has major regulatory roles in the circadian system.

The molecular mechanism of circadian transcriptional regulation is best understood for the *DBP* gene, highly expressed and strongly rhythmic in the SCN and peripheral tissues such as the liver (Lopez-Molina *et al*., 1997; Yan *et al*., 2000), which involves the rhythmic binding of CLOCK/BMAL1 heterodimers to E-box elements in an intron 2 enhancer in coordination with rhythmic changes in histone modification (Ripperger *et al*., 2000; Ripperger & Schibler 2006; Kiyohara *et al*., 2008). Additionally, CRY1 can also be recruited to the non-canonical E-box in the *DBP* and cause delay phrase of *DBP* expression in liver (Stratmann *et al*., 2010). In this study, we further dissected the *cis*-acting *DBP* enhancer and identified a significant involvement of NRF1, in which the NRF1 site closely flanks the two E-boxes in intron 2 of *DBP* [**Figure 3.1(g)**]. Indeed, NRF1 affects the amplitude of transcriptional activity due to *DBP* enhancer activity to the same degree as the effect triggered by CLOCK/BMAL1 heterodimers in unstimulated NIH3T3 cells [**Figure 3.4(f) & Figure 3.5**]. Although in transfection experiments the combination of NRF1, CLOCK and BMAL1 was not dramatically additive, it is possible that the *DBP*

transcriptional capacity in the luciferase reporter system is saturated by NRF1 alone or CLOCK-BMAL1 alone. Alternatively, NRF1 and CLOCK/BMAL1 may have independent roles in activation of *DBP* enhancer function. The latter seems unlikely given that we have identified NRF1 and the active CLOCK transcription factor in a molecular complex, making it likely that NRF1 and CLOCK function together in a complementary manner. Interestingly, the amplitude of transcriptional activity (Dibner *et al*., 2009; O'Neill, 2009) plays a role in the period of the circadian clock, so NRF1 may contribute to this aspect of circadian function by its ability to strongly activate transcription through enhancer and promoter mechanisms.

By protein immunoprecipitation studies we have shown that NRF1 and CLOCK/BMAL1 interact in a molecular complex, and it is striking that NRF1 preferentially interacts with the phosphorylated, active isoform of CLOCK in the nucleus [**Figure 5(c)**; Lee *et al*., 2001; Tamaru *et al*., 2003; Shim *et al*., 2007]. It is known that the status of CLOCK phosphorylation is dynamic, with phosphorylation of CLOCK associated with its translocation from the cytoplasm to the nucleus (Kondratov *et al*., 2003; Shim *et al*., 2007) and an enhancement of its E-box binding ability, whereas hyperphosphorylation decreases protein stability of CLOCK (Yoshitane *et al*., 2009; Spengler *et al*., 2009). Furthermore, many factors can transiently interact with CLOCK/BMAL1 for specific functions, for example, CIPC-mediated CLOCK phosphorylation (Yoshitane *et al*., 2009), SIRT1-mediated deacetylation of BMAL1 and PER2 (Asher *et al*., 2008; Nakahata *et al*., 2008; Masri & Sassone-Corsi, 2010), and MLL1 (mixed lineage leukemia-1)-mediated methylation of H3K4 (Masri & Sassone-Corsi, 2010). Therefore, it is plausible that the interaction between NRF1 and CLOCK/BMAL1 also occurs in a transient manner at the promoter or enhancer elements of a subset of genes involved in circadian rhythms.

Interestingly, our data showed that peak levels of NRF1 mRNA and protein oscillate with an ~16 hr ultradian rhythm (~1.5th harmonic cycle, Hughes *et al*., 2009) in serum-shocked NIH3T3 cells [**Figure 3.7(c)**]. It is not surprising that NRF1 levels oscillate, as in other work (see **Chapter 4**), we found that NRF1 is autoregulated by a feed-forward mechanism, although the mechanism of negative feedback regulation recquisite for an oscillatory system is not known. One candidate would be a repressive transcriptional mechanism (see **Chapter 4**). Another mechanism that could negatively regulate *NRF1* mRNA and protein levels would be miRNA targeting, and our recent studies have found that miR-182 and miR-96 are involved in down-regulation of NRF1 (B. J. Henson, R. D. Nicholls, unpublished data). This is intriguing as the miR-182/miR-96 family of miRNAs has been suggested to show circadian expression in retinal neurons (Xu *et al*., 2007) although more recent studies suggest that the oscillatory activation of miR-182/miR-96 in retinal photoreceptor neurons is due to activation by light with rapid turnover in the dark (Krol *et al*., 2010). These and additional mechanisms acting in retinal (Becker *et al*., 1998) and/or SCN neurons could thus play a role in regulation of NRF1 and subsequent circadian regulation of downstream NRF1 target genes. Combined with our data showing that NRF1 targets include genes from both positive and negative feedback loops (**Table 3.3**), we propose that the circadian system can interpret the oscillating levels of NRF1 and its interaction with the CLOCK/BMAL1 complex to regulate the affinity and binding at *cis*-regulatory elements of target genes to integrate the regulatory networks of both the positive and negative circadian feedback loops.

Genome-wide microarray analyses have shown that 10-15% of genes are CCGs with circadian expression and that many of these genes are involved in metabolism and energy balance (Reppert & Weaver, 2002; Mendoza, 2007; Masri & Sassone-Corsi, 2010). Interestingly, energy balance in the body is sensed and regulated by the AMP-activated protein kinase (AMPK, Hardie,

2008; Spasic *et al*., 2009; Richter *et al*., 2009; Lim *et al*., 2010), which regulates the circadian clock through phosphorylation and stability of CRY1 and entrains circadian function to metabolism (Lamia *et al*., 2009; Suter & Schibler, 2009). AMPK also activates NRF1-pathways (Bergeron *et al*., 2001; Garcia-Roves *et al*., 2008), and provides a link between energy balance, diabetes (Hardie, 2008), exercise (Ojuka *et al*., 2004), and NRF1 levels and activity (Timmons *et al*., 2006; Wright *et al*., 2007). Additionally, PPARGC1A is one of several co-activators for NRF1, particularly for nuclear genes encoding mitochondrial functions (Scarpulla, 2008), and PPARGC1A is known to play a key role in integration of circadian function (Liu *et al*., 2007) and metabolism (Cunningham *et al*., 2007). Collectively, this provides a link of NRF1 to energy balance and metabolism, each of which can critically influence circadian rhythms.

In vertebrates, light entrains the developing circadian clock via photoreceptive retinal ganglion cells that express melanopsin and that project to the SCN (Vallone *et al*., 2007). Intriguingly, mutation of *NRF1* ortholog in zebrafish demonstrated an essential role in development of all types of photoreceptors (Becker *et al*., 1998). The *NRF1* ortholog in fly (*EWG*) is also essential for neuronal development (DeSimone & White, 1993). Supporting evidence from the Allen brain atlas (Jones *et al*., 2009) by *in situ* hybridization shows enriched expression of *Nrf1* in the SCN of the hypothalamus in mouse, as well as hypothalamic nuclei involved in the regulation of feeding, and other non-hypothalamic neuronal structures. This observation, combined with our data showing NRF1 contributes to enhancer function of the SCN-specific *DBP* enhancer (Ripperger et al., 2000), as well as other key genes for SCN circadian function such as *CLOCK*, *PER1*, *CRY1*, *NR1D1*, and others, supports a hypothesis for a role of NRF1 in gene regulation of the central pacemaker of circadian rhythms. Future studies will determine the roles

and mechanisms of action of NRF1 in establishment and/or function of neural and molecular pathways underlying circadian rhythms.

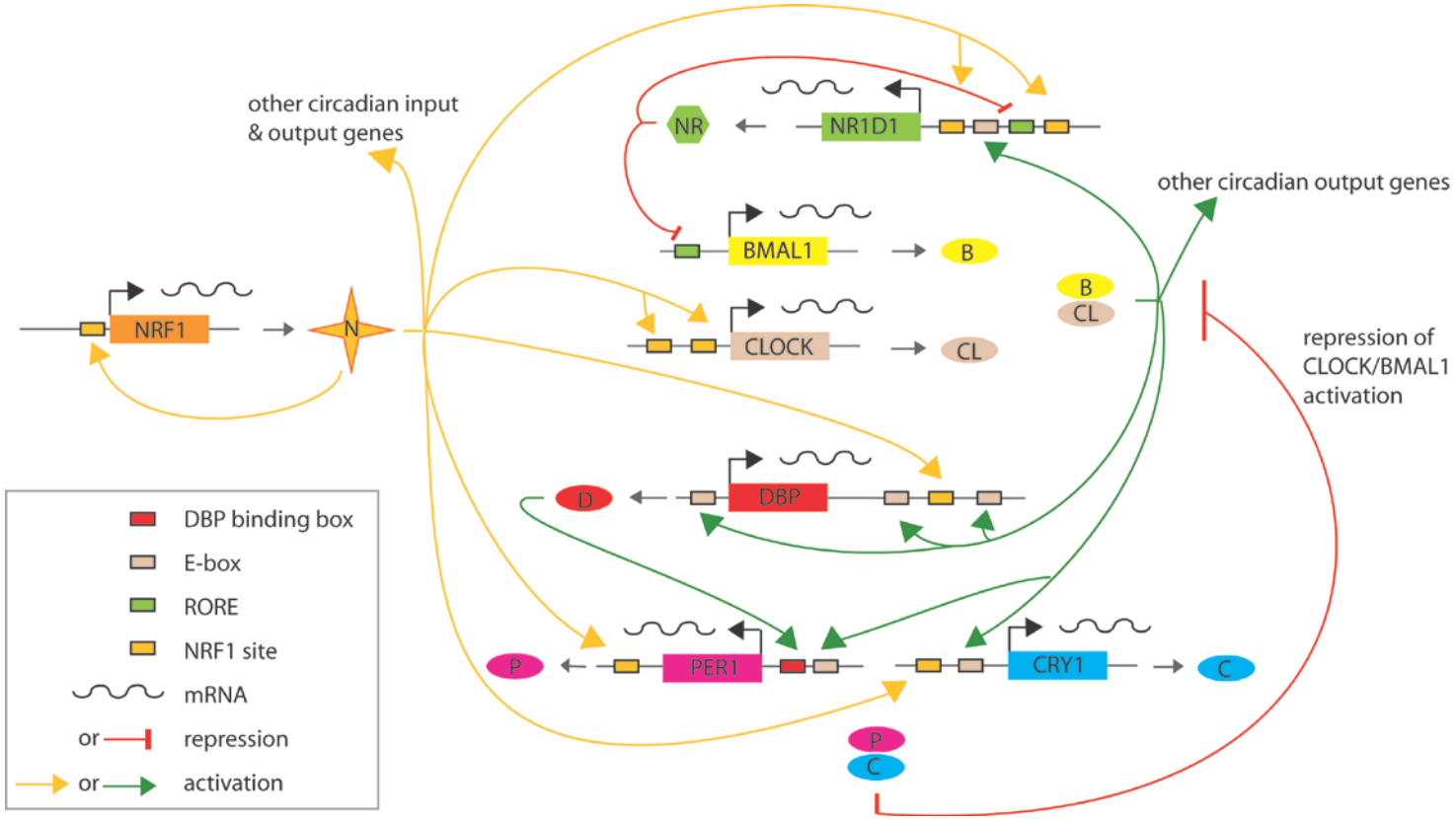


Figure 3.8. Summary of NRF1 regulation in the circadian system. The model depicts the canonical clock mechanism involving positive and negative feedback loops comprised of CLOCK/BMAL1 heterodimers binding to E-box elements in target genes and subsequent repression of these pathways by PER/CRY heterodimers. NR1D1 provides an ancillary feedback loop, while DBP encodes an output regulatory TF and also regulates PER1. Post-translational regulation by phosphorylation and protein degradation are not shown for simplicity. NRF1 regulation occurs for many components of the circadian regulatory hierarchy.

4.0 ADDITIONAL MECHANISMS AND SYSTEMS OF NRF1 REGULATION

4.1 INTRODUCTION

4.1.1 Hereditary spastic paraplegia

Hereditary spastic paraplegias (HSP) are neurodegenerative disorders characterized primarily by progressive spasticity in the lower limbs due to axonal degeneration of the corticospinal tract (CST). Other neural-related clinical features include progressive symmetric spasticity of lower extremities, often but not always with muscle weakness, and increased stiffness, hyperreflexia, extensor plantar responses and, in some cases, bladder disturbances and vibratory sense impairment (Depienne *et al*., 2007; Fink 2009). Dominant inheritance accounts for ~70% of HSP and mutations in one gene, *SPAST* (*SPG4*), occur in about 40% of these cases. However, to date, 45 autosomal dominant, recessive and X-linked gene loci are chromosomally linked to HSP pathogenesis, with etiological gene mutations identified for only 22 of these (Depienne *et al*., 2007; Salinas *et al*., 2008; Dion *et al*., 2009; Fink, 2009). Selection of new candidate genes that affect function and trafficking in long axons is difficult as known hereditary spastic paraplegia (HSP) genes encode proteins involved in a multitude of biochemical pathways including known or potential roles in mitochondria, microtubules, anterograde transport, endosomal, endoplasmic reticulum (ER) and Golgi, as well as additional functions such as cytoplasmic, gap junction, and myelin sheath or Schwann cell function (Salinas *et al*., 2008; Dion *et al*., 2009; Park *et al*., 2010).

Our knowledge of *cis*-binding motifs and role of NRF1 in transcriptional regulation in the PWS region (**Chapter 2**) provided a basis for initially examining *SPG6* (*NIPA1*, on human chromosome 15q11.2), followed by the *Spastin* (*SPG4*) gene promoter since *SPG4* is most frequently involved in spastic paraplegia. Having identified potential NRF1 binding sites in the promoters of these two genes (see Results), we extended our analyses to all 22 cloned HSP loci. We hypothesize that HSP loci are co-regulated by a common GRN in order to determine proper spatiotemporal expression patterns in the motor cortex to accomplish specific CST functions.

4.1.2 NRF1 autoregulation

NRF1 is known to be crucial for the expression of nuclear genes that encode mitochondrial products (Scarpulla, 2008), genes in cell cycle regulation (Morrish *et al*., 2003; Cam *et al*., 2004), development (Chen *et al*., 1997; Efiok & Safer, 2000; Moriuchi *et al*., 1997; Solecki *et al*., 2000) and neural functions (Chang & Huang, 2004; Smith *et al*., 2004; Chang *et al*., 2005; Yang *et al*., 2006). Indeed, loss of Nrf1 function is lethal across the animal kingdom (**Chapter 1.1.1**). Previous reports have shown that some TFs are autoregulated by binding to their own promoters. For example, NR1D1 has a Rev-erbα response element (RORE) on its own promoter [**Figure 3.1(e)**], which has been shown to mediate transcriptional repression when NR1D1 protein level goes up in an *in vitro* system, implying this gene is potentially autoregulated (Adelmant *et al*., 1996). YY1 has a cluster of YY1 binding sites on its intron 1 as a transcriptional activator (Kim *et al*., 2009). When exogenous YY1 was overexpressed in an induced cell line, endogenous YY1 protein level went down to maintain homeostasis through regulation by the cluster of YY1 sites intron 1, proving the mechanism of YY1 autoregulation (Kim *et al*., 2009). These data suggested a TF autoregulatory system is functioned by a transcriptional feedback loop

to repress its own expression. Intriguingly, we found two highly conserved NRF1 sites in the 5' regulatory region and one site in intron 1 of the *NRF1* gene (**Figure 4.1**). All putative NRF1 sites are highly conserved from fish to human (**Figure 4.1**). Therefore, we hypothesize that NRF1 autoregulates its own transcriptional activity.

4.1.3 NRF1 binding site studies

As mentioned in Chapter **1.1.4**, several genome-wide studies by bioinformatics analysis or experiments (ChIP-chip) have revealed that approximately 5-6% of the genes are regulated by NRF1. Functional NRF1 binding sites have been confirmed experimentally with sequence variance of 1 nucleotide in either GCGC and/or 1 nucleotide in the AY segment (Stefan *et al*., in preparation, **Figure 2.2 & 3.1**). These studies also suggested that multiple NRF1 binding sites may also increase binding affinity by cooperative binding even if individual recognition motifs may not be optimal.

To further study the parameters that affect NRF1 binding ability, an artificial model has been designed. In this model, six minigenes have been synthesized in pIDTSMART vectors (IDT), each containing 10, 8, 6, 4, 2, or 1 copies of NRF1 consensus recognition sites. These minigenes were subcloned from the pIDTSMART plasmids into the promoter position of a modified pGL3basic luciferase reporter vector. Subsequently, these series of pGL3basic-derived vectors were transfected into SK-N-SH neuroblastoma cells to compare the luciferase activities. These studies will not only improve bioinformatics methods for identification of functional NRF1 sites genome-wide, in any animal species, but will also provide critical knowledge and reagents to aid structural analyses of NRF1 as well as the generation of novel reagents for future *in vivo* functional studies.

Figure 4.1. Multi-sequence alignment of the *NRF1* promoter-exon 1-intron 1 region. NRF1 sites are highlighted in bold red. A YY1 site is highlighted in bold pink. Sp1 sites are highlighted in bold purple. The exon/intron boundary is highlighted in bold blue.

4.2 MATERIALS AND METHODS

4.2.1 Bioinformatics and phylogenetic analyses

NRF1 sites were predicted using bioinformatics by matches on either strand to the consensus binding motif 5'-yGCGCANGCGCr-3', allowing one mismatch in one but not both GCGC motifs (y = t or c; r = a or g; neither of these two positions is required but generates a more optimal motif). Sites with one mismatch in one GCGC motif are defined as an NRF1-like site (L); various substitutions lead to differing affinity for NRF1 as determined experimentally (e.g., ACGC remains high affinity with strong binding of NRF1, but GCCC has little or no affinity, although multiple NRF1 or NRF1-like sites in a promoter can increase the binding extent at even weaker sites).

4.2.2 Gene expression studies

RNA was extracted from human SK-N-SH neuroblastoma cells with Trizol (Invitrogen) according to the manufactuer's protocol. DNase I treatment was performed to remove DNA contaminants from each RNA sample. Based on RNA concentration, determined using a spectrophotometer, 1 µg of RNA from each sample was transcribed into cDNA by SuperScript® III reverse transcriptase according to the manufacturer's protocol (Invitrogen). Regular PCR was performed to test gene expression of a subset of HSP genes using primers from **Table 4.1**.

Table 4.1. Human QPCR primers for HSP genes.

Target gene (primer)[a]	Primer number	Exon	Primer sequences
SPG3A F	RN3632	7-8	5'-TCAAGGTCTCAGGGAACCAGC-3'
SPG3A R	RN3633	8	5'-CCAGGATGAGGTAGCAGAAAAC-3'
SPG4 F	RN3430	15	5'-GACTGATGGATACTCAGGAAG-3'
SPG4 R	RN3431	16	5'-GGCAGACATATTCTTCACCTG-3'
SPG5 F	RN3613	4	5'-TTTCCCATCCACCTCACCAG-3'
SPG5 R	RN3614	5	5'-CCCGGTCTCTGAACTGAGAG-3'
SPG6 F	RN3024	4	5'-GCTCCGTCGTGCTGATTAT-3'
SPG6 R	RN3025	5	5'-AGGTAGCCCACAAACACTGG-3'
SPG7 F	RN3428	15	5'-GGTGAAGCAGTTTGGGATGG-3'
SPG7 R	RN3429	16	5'-CTTGGCCACCAGCAGTCTTG-3'
SPG8 F	RN3432	27	5'-GCCAGTTTATCTGCTCCACG-3'
SPG8 R	RN3433	28	5'-GCACCCACAACATCTGCAGG-3'
SPG10 F	RN3634	9-10	5'-CTGGCTGAGGGCACTAAAAG-3'
SPG10 R	RN3635	10	5'-TGAGCAACAGATGAACATAGTC-3'
SPG11 F	RN3624	7	5'-GCTGATGGACTGTATTCTGG-3'
SPG11 R	RN3625	8	5'-AATCTGGAACAAAATCGTAGGC-3'
SPG13 F	RN3434	10	5'-GATGCATTCCAGCCTTGGAC-3'
SPG13 R	RN3435	11	5'-CAAAGATCCTTCAACACCTGC-3'
SPG15 F	RN3636	4	5'-TTGTACTGGAGAAATGGTTGGC-3'
SPG15 R	RN3637	5	5'-TCTGACAATAAAAGAAACTCAAGC-3'
SPG17 F	RN3638	5	5'-CCTCCTGCTATTTGGCTTTGC-3'
SPG17 R	RN3639	6	5'-CTGTGGATCTCAATGATCGCTC-3'
SPG20 F	RN3438	7	5'-CATGTCAAGAAGCATGGAAGC-3'
SPG20 R	RN3439	8	5'-AGCTGCACATTCCAATCCTTG-3'
SPG21 F	RN3640	3	5'-GCTCTGACTGGATGGGGTTAC-3'
SPG21 R	RN3641	4	5'-CTGAATCCATCACAGAACTCG-3'
SPG31 F	RN3642	3	5'-GCACTTTTCACCACAGCAGAG-3'
SPG31 R	RN3643	4	5'-TTAGTTCATAATAGAATGGAAACC-3'
SPG33 F	RN3436	7	5'-CGGGTTCCTGAGCAAGAATG-3'
SPG33 R	RN3437	8	5'-TGAGAAGGTGGCCGAGCAG-3'

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

4.2.3 Chromatin immunoprecipitation (ChIP)

Approximately 1×10^6 SK-N-SH neuroblastoma cells or marsupial cells were plated on 35 cm^2 plates with regular medium [medium for SK-N-SH cells: Alpha modified MEM + 10% FBS + 1% penicillin-streptomycin (P/S) + 1% 100 mM L-glutamine + 1% nonessential amino acid + 1% sodium pyruvate; medium for marsupial cells: DMEM+10% FBS + 1% penicillin-streptomycin (P/S)]. When cells reached 70-80% confluence, formaldehyde was added into the regular media to make the final concentration of 1% and the plates were incubated at 37°C for 10 minutes to cross link protein to DNA. After washing with 5 ml ice cold PBS once, another 5 ml ice cold PBS was added, cells were scraped from the plates by a cell lifter, put into a 15 ml tube, centrifuged, re-suspended in SDS lysis buffer (ChIP assay kit, Millipore), and sonicated to shear DNA. Samples were precleared with protein G-agarose/salmon sperm beads (Millipore). Protein-DNA complexes were immunoprecipitated with anti-NRF1 (from Dr. Daniel Raines, Smith *et al*., 2004) or anti-YY1 (Santa Cruz Biotechnology, sc-1703X). Complexes were collected with Protein G agarose/salmon sperm beads and washed. Protein-DNA complexes were eluted off the beads and cross-links were reversed by incubation with NaCl at 65ºC overnight. On the next day, DNA was recovered by phenol-chloroform extraction and precipitated by ethanol. PCR was performed using the recovered immunoprecipitated DNA materials as templates and primers from **Table 4.2** and **Table 4.3**. The PCR products were run on an agarose gel to examine the results.

4.2.4 *NRF1* siRNA assays

NRF1 siRNA primers were designed (Brummelkamp *et al*., 2002; Smith *et al*., 2004), cloned into the pSUPER siRNA vector and then sequenced to confirm the correct structure. SK-

N- SH cells were sub-cultured onto 75 cm^2 flasks 1 day before the nucleofection and reached 80-90% confluence. On the day of nucleofection, 4 μg of pSUPER-NRF1 DNA were transfected into 1×10^6 SK-N-SH cells using an Amaxa cuvette (Lonza) with Nucleofector® (Lonza) and the transfected cells were plated onto a 6-well plate. 24 hr after nucleofection, RNA was extracted with Trizol and treated by DNase I to remove genomic DNA contaminants. 1 μg of RNA from each sample was reverse transcribed into cDNA using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacture's instruction. PCR was performed using primers for each gene (**Table 4.1**) to examine gene expression with *GAPDH* as a negative control and *NRF1* as a positive control to ensure effective down regulation by siRNA.

Table 4.2. Human ChIP primers for HSP genes.

Target gene (primer)[a]	Primer number	Primer sequences
SPG5 F	RN3511	5'-TTGCTTGTCCGGCGCACCAG -3'
SPG5 R	RN3512	5'-TCCCCTCGCCATCTGGGTC-3'
SPG6 F	RN3630	5'-CCCCTCTTCCTGCTCCTCC-3'
SPG6 R	RN3631	5'-CACCAGGCTCGACACGACG-3'
SPG7 F	RN3464	5'-TCAGGCAGCCACGAGGTAGAC-3'
SPG7 R	RN3465	5'-AGCAGCAGCACGGCCATGTTG-3'
SPG11 F	RN3622	5'-ACGAATGGAATCGACCGGAG-3'
SPG11 R	RN3623	5'-CTTCCTCTGCAGCCATCTTG-3'

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

Table 4.3. ChIP primers for the *NRF1* promoter.

Target gene (primer)[a]	Primer number	Primer sequences
Human *NRF1*promoter F	RN3511	5'-TTGCTTGTCCGGCGCACCAG -3'
Human *NRF1* promoter R	RN3512	5'-TCCCCTCGCCATCTGGGTC-3'
Human *SNURF-SNRPN* enhancer F	RN2683	5'-CAGGTCTTGGAAGGCTATGTCG-3'
Human *SNURF-SNRPN* enhancer R	RN2684	5'-CTCCCCACTGGCGGCTCTAC-3'
Marsupial *Nrf1* promoter F	RN3464	5'-TCAGGCAGCCACGAGGTAGAC-3'
Marsupial *Nrf1* promoter R	RN3465	5'-AGCAGCAGCACGGCCATGTTG-3'
Marsupial *Snrpb* promoter F	RN3751	5'-AATAAGAAACAATGAATATGTGAGG-3'
Marsupial *Snrpb* promoter R	RN3752	5'-CACGACAAAACGGTCCGACTG-3'
Marsupial *Tfam* promoter F	RN3622	5'-ACGAATGGAATCGACCGGAG-3'
Marsupial *Tfam* promoter R	RN3623	5'-CTTCCTCTGCAGCCATCTTG-3'

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

4.2.5 Reporter constructs and luciferase reporter assays

The minimal promoters of *SPG4* and *SPG6* were cloned by PCR into a pGL3-enhancer (pGL3e) luciferase vector using primers from **Table 4.4.** Either the pGL3e-*SPG4* or -*SPG6* promoter construct was transfected into mammalian cells with 1) control siRNA; 2) siRNA targeting luciferase mRNA; or 3) siRNA targeting *NRF1* mRNA.

Based on sequence alignments of conserved promoter sequence of *NRF1*, luciferase reporter constructs were made by PCR cloning using primers in **Table 4.5** to clone the minimal promoter sequence using genomic DNA from human SK-N-SH cells as template. PCR products were digested with *Bgl*II and *Hin*dIII for promoters or *Bam*HI and *Sal*I for enhancers, and the respective products ligated directionally into the pGL3basic vector (Promega). Site directed mutations were performed using primers in **Table 4.5** with the GeneTailor™ Site-Directed

Mutagenesis Kit (Invitrogen) according to manufacturer's instruction, and the mutated products were amplified by Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). Each construct was sequenced to verify correct inserts.

To study the parameters that affect NRF1 binding ability, six minigenes were synthesized in pIDTSMART vectors (by Integrated DNA Technology, Inc.), each containing 10, 8, 6, 4, 2, or 1 copies of NRF1 consensus recognition sites (**Table 4.6**). These minigenes were subcloned from the pIDTSMART plasmids into the promoter position of a modified pGL3basic luciferase reporter vector (**Figure 4.6**).

SK-N-SH cells were grown in a 6-well cell culture plate at a concentration of 1.8×10^5 cells/well and each well cultured in 2 ml cell medium. When the cells reached 90% confluence, 1 μg of each construct was transfected along with the pRL vector (Promega), a second luciferase vector used for normalization in the Dual-Luciferase® Reporter Assay System (Promega), with Lipofectamine 2000 (Invitrogen) into the cells. 24 hr after transfection, cell medium was removed from the cell culture, and washed once with PBS. 500 μl passive lysis buffer (Promega) was applied to each well and the culture plate was rocked at room temperature for 15 min. The lysates were then collected into a 1.5 ml tube and 100 μl LARII (Promega; freshly prepared for each luciferase assay) was added. The first luciferase activity reading was measured on a 20/20n luminometer (Turner Biosystem). Subsequently, 100 μl Stop & Glo® reagent (Promega) was added which allows the measurement of a second luciferase activity (*Renilla*) reading to normalize the results. The luciferase assay was repeated independently three times to test statistical significance by a student's t-test.

Table 4.4. Primers for *SPG4* and *SPG6* promoter luciferase constructs.

Target gene (primer)[a]	Primer number	Primer sequences[b]
Human *Spastin* (*SPG4*) pr F	RN3470	5'-GCTT**ACGCGT**TGAGCCGAACTGCACATTGG-3'
Human *Spastin* (*SPG4*) pr R	RN3471	5'-CATA**CTCGAG**GTCTCAGGAGCTCCGCACTG-3'
Human *NIPA1* (*SPG6*) pr F	RN3472	5'-GCTT**ACGCGT**GCCCGCGCCTCCCGGTCACC-3'
Human *NIPA1* (*SPG6*) pr R	RN3473	5'-CATA**CTCGAG**GCAGCTGCAGTCCCCATTCC-3'

[a] Abbreviations: pr: promoter; F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

[b] Enzyme digestion sites are in bold and underlined.

Table 4.5. Primers for *NRF1* promoter luciferase constructs.

Target gene (primer)[a]	Primer number	Primer sequences
NRF1 sub-cloning primers into pGL3-basic promoter site F	RN3622	5'-TCCA**AGATCT**GACCTCCTCATCATGGCGG-3'
NRF1 sub-cloning primers into pGL3-basic promoter site R	RN3623	5'-AATT**AAGCTT**CTCAGAGCGGCTGCGCTAC-3'
NRF1 promoter YY1 mutation minigene for luciferase reporter assays	RN3846	5'-**AGATCT**gacctcctcatcatggcggcggccgggcggggaagccgggccgtgcgt cgcgtgcgtgccctctccctcccctcccctcctcggcggcggcggcggcggcagaa gcggcagcgct**cgcAaAAT**ccgctggtggcaggaggctgcgaggagccggcgcggtc gcagtctccacg**gcgcaggccca**cggta**gcgcagccgc**tctgag**AAGCTT**-3'
NRF1 promoter 5' NRF1-like mutation minigene for luciferase reporter assays	RN3847	5'-**AGATCT**gacctcctcatcatggcggcggccgggcggggaagccgggccgtgcgt cgcgtgcgtgccctctccctcccctcccctcctcggcggcggcggcggcggcagaa gcggcagcgct**cgccattg**ccgctggtggcaggaggctgcgaggagccggcgcggtc gcagtctccacg**gAATaggAcca**cggta**gcgcagccgc**tctgag**AAGCTT**-3'
NRF1 promoter 3' NRF1-like mutation minigene for luciferase reporter assays	RN3848	5'-**AGATCT**gacctcctcatcatggcggcggccgggcggggaagccgggccgtgcgt cgcgtgcgtgccctctccctcccctcccctcctcggcggcggcggcggcggcagaa gcggcagcgct**cgccattg**ccgctggtggcaggaggctgcgaggagccggcgcggtc gcagtctccacg**gcgcaggccca**cggta**gAATagccAc**tctgag**AAGCTT**-3'
NRF1 promoter 5' & 3' NRF1-like mutations minigene for luciferase reporter assays	RN3849	5'-**AGATCT**gacctcctcatcatggcggcggccgggcggggaagccgggccgtgcgt cgcgtgcgtgccctctccctcccctcccctcctcggcggcggcggcggcggcagaa gcggcagcgct**cgccattg**ccgctggtggcaggaggctgcgaggagccggcgcggtc gcagtctccacg**gAATaggAcca**cggta**gAATagccAc**tctgag**AAGCTT**-3'

[a] Abbreviations: F: forward PCR primer; R: reverse PCR primer. Gene sequences were obtained from the Ensembl genome browser (http://www.ensembl.org/Homo_sapiens/Info/Index).

[b] Enzyme digestion sites are in bold and underlined. YY1 site is in bold pink. NRF1 site is in bold red. Mutated nucleotides are in uppercase.

Table 4.6. Minigenes for NRF1 tandem arrays in luciferase constructs.

Minigenes with copy number[a]	Primer number	Primer sequences[b]
NRF1 site tandem array (10 copies) in a 230-bp mini gene	RN3755	5′-TTAA**CTCGAG**CGCGCATGCGCACCTCTGGGCC**GTCGCATGCGCA**CCAGTCTAGC**CG** **CGCAGGCGCG**CTAGTCAGGC**TGCGCATGCGCG**CCATTGCCTG**TGCGCAAGCGCG**CTACT CGCAC**TGCGCATGCGCA**CCTTTGCATG**GGCGCCTGCGCA**CAATGGTCTG**CGCGCAGGCG** **CA**CACGTCAGAC**TGCGCAGGCGTG**CACGTCGGGC**GGCGCATGCGCA****AGATCT**TGTG-3′
NRF1 site tandem array (8 copies) in a 186-bp mini gene	RN3756	5′-TTAA**CTCGAG**CGCGCATGCGCACCTCTGGGCC**GTCGCATGCGCA**CCAGTCTAGC**CG** **CGCAGGCGCG**CTAGTCAGGC**TGCGCATGCGCG**CCATTGCCTG**TGCGCAAGCGCG**CTACT CGCAC**TGCGCATGCGCA**CCTTTGCATG**GGCGCCTGCGCA**CAATGGTCTG**CGCGCAGGCG** **CA****AGATCT**TGTG-3′
NRF1 site tandem array (6 copies) in a 142-bp mini gene	RN3757	5′-TTAA**CTCGAG**CGCGCATGCGCACCTCTGGGCC**GTCGCATGCGCA**CCAGTCTAGC**CG** **CGCAGGCGCG**CTAGTCAGGC**TGCGCATGCGCG**CCATTGCCTG**TGCGCAAGCGCG**CTACT CGCAC**TGCGCATGCGCA****AGATCT**TGTG-3′
NRF1 site tandem array (4 copies) in a 98-bp mini gene	RN3758	5′-TTAA**CTCGAG**CGCGCATGCGCACCTCTGGGCC**GTCGCATGCGCA**CCAGTCTAGC**CG** **CGCAGGCGCG**CTAGTCAGGC**TGCGCATGCGCG****AGATCT**TGTG-3′
NRF1 site tandem array (2 copies) in a 54-bp mini gene	RN3759	5′-TTAA**CTCGAG**CGCGCATGCGCACCTCTGGGCC**GTCGCATGCGCA****AGATCT**TGTG- 3′
NRF1 site tandem array (1 copy) in a 32-bp mini gene	RN3760	5′-TTAA**CTCGAG**CGCGCATGCGCA****AGATCT**TGTG-3′

[a] NRF1 binding sequences are experimentally confirmed NRF1 binding sites or high affinity sites based on the consensus sequence: 5'-YGCGCANGCGCR-3'. DNase I footprint assay showed that at least 4 nucleotides on flanking sequence was needed for NRF1 binding (Virbasius & Scarpulla, 1994).

[b] NRF1 sites are in bold red. Enzyme digestion sites are in bold and underlined.

4.3 RESULTS

4.3.1 NRF1 regulates hereditary spastic paraplegia genes *in trans*

In this study, we have identified potential NRF1 binding sites in the promoters a total of 10 known HSP loci with putative NRF1 binding sites in the 5' regulatory region (**Table 4.7**). Importantly, the putative NRF1 binding sites in the promoters of most of the HSP loci were evolutionary conserved in all eutherian mammals, and for some sites in a marsupial (*M. domestica*) and a monotreme (platypus) (examples are shown in **Figure 4.2** for *SPG4*, *SPG5*, *SPG6* and *SPG7*). Evolutionary maintenance of so-called "phylogenetic footprints" provides strong evidence that a DNA-binding protein recognizes that sequence. In several of the HSP gene promoters, such as for *SPG6* and *SPG7*, putative NRF1 and Sp1 (a TF binding at most "CpG-island" of mammalian gene promoters) motifs were the only predicted conserved binding sites and for others, such as *SPG5* and *SPG4*, the putative NRF1 binding site(s) were the main conserved binding sites, attesting to the likely significance of NRF1 regulation.

To show that NRF1 physically binds *in vivo* at these regulatory elements, we used ChIP in a human neuroblastoma cell line, SK-N-SH, that robustly expresses mRNA for all investigated HSP genes (**Figure 4.3;** data not shown). The ChIP assay confirmed that NRF1 binds specifically and robustly to the promoters at all predicted sites for HSP genes in human SK-N-SH cells (examples are shown in **Figure 4.4**, summarized in **Table 4.5**). Similarly, ChIP assays were performed in mouse Neuro2A cells and NRF1 binds robustly to all predicted sites for mouse orthologs of HSP genes (data not shown). NRF1 regulation of HSP loci within neuronal-like cells was further confirmed by knocking down the steady-state level of endogenous *NRF1* mRNA using silencing RNA (siRNA) studies. The siRNA treatment was effective, knocking down the

NRF1 mRNA level dramatically but not affecting the mRNA level of a control gene, *GAPDH* (**Figure 4.5**). Indeed, all tested HSP loci having NRF1 motifs that bind the TF in ChIP assays demonstrated a major reduction in mRNA levels in the *NRF1* siRNA-treated cells (**Figure 4.5**).

Utilizing luciferase reporter vectors, further assays of transcriptional promoter function have been employed to molecularly dissect the transcriptional mechanisms for HSP gene promoters. Functional promoters will drive transcription, with the degree of light produced a marker of the amount of luciferase enzyme produced and hence of transcriptional activation levels. We did this for the evolutionary conserved segments of the *SPG4* and *SPG6* promoters (**Figure 4.6**), with high levels of promoter activity found [**Figure 4.6(b) & (c),** control siRNA columns]. Using co-transfection with a siRNA targeting the luciferase mRNA, promoter activity was significantly down-regulated [**Figure 4.6(b), (c)**], while both *SPG4* and *SPG6* promoter activity was essentially abolished using siRNA targeting the endogenous *NRF1* mRNA [**Figure 4.6(b), (c)**].These results confirm the phylogenetic predictions, that NRF1 provides the major regulatory activity at these promoters.

Table 4.7. Summary of HSP loci with NRF1 regulation.

HSP locus (Gene)	Human Chr[a]	Presence of putative NRF1 site by bioinformatics[b]	Function	ChIP confirmation in SK-N-SH cells
SPG4 (*SPASTIN*)	2p22	+	Cytosolic microtubule severing protein, with AAA and MIT domains	Yes
SPG5 (*CYP7B1*)	8q12.3	+ (intron 1)	Cytochrome p450 enzyme, cholesterol and steroid metabolism	Yes
SPG6 (*NIPA1*)	15q11.2	+	Transporter; in ER and endosome membranes	Yes
SPG7 (*PARAPLEGIN*)	16q24.3	+++	Mitochondrial protein, AAA ATPase domain	Yes
SPG8[c] (*STRUMPELLIN*)	8q24	+	Protein function unknown, contains spectrin domains, involved in motor-neuron outgrowth	Yes
SPG11 (*SPATACSIN*)	15q15.3-q21.1	L[a]	Not known (proposed TM domains not verified by our analyses)	Yes
SPG13 (*HSP60*)	2q24-q34	++L	Mitochondrial chaperonin, heat shock protein	Yes (proximal and distal)
SPG20 (*SPARTIN*)	13q12.3	+	MIT domain, endosomal protein	Yes
SPG33 (*ZFYVE27*)	10q24.2	+	Promotes neurite formation, involved in membrane traffic events	Yes
SPG42 (*SLC22A1*)	3q25.31	_[d]	Acetyl-CoA transporter	Not tested

[a] Abbreviations: chr: chromosome; L: NRF1-like site (sites with one mismatch in one GCGC motif are defined as NRF1 like site).

[b] All confirmation of NRF1 sites was by ChIP analysis using human SK-N-SH and mouse Neuro2A neuroblastoma cell lines, and for *SPG4* and *SPG6* also by luciferase reporter promoter analyses. In addition, NRF1 regulation of all these genes was seen using siRNA that targeted and knocked down the level of *NRF1* mRNA in human SK-N-SH neuroblastoma cells.

[c] No NRF1 site occurs in the 5' regulatory region in rodents; the NRF1 site in human *SPG8* and NRF1-like site in *SPG11* are conserved in all other non-rodent sequenced mammalian genomes.

[d] No NRF1 site in the 5' regulatory region in primates; a site in mouse *Spg42* is conserved in all other sequenced mammalian genomes.

(a) CLUSTAL 2.0.10 multiple sequence alignment for the conserved part of the *SPG4* (*SPASTIN*) promoter

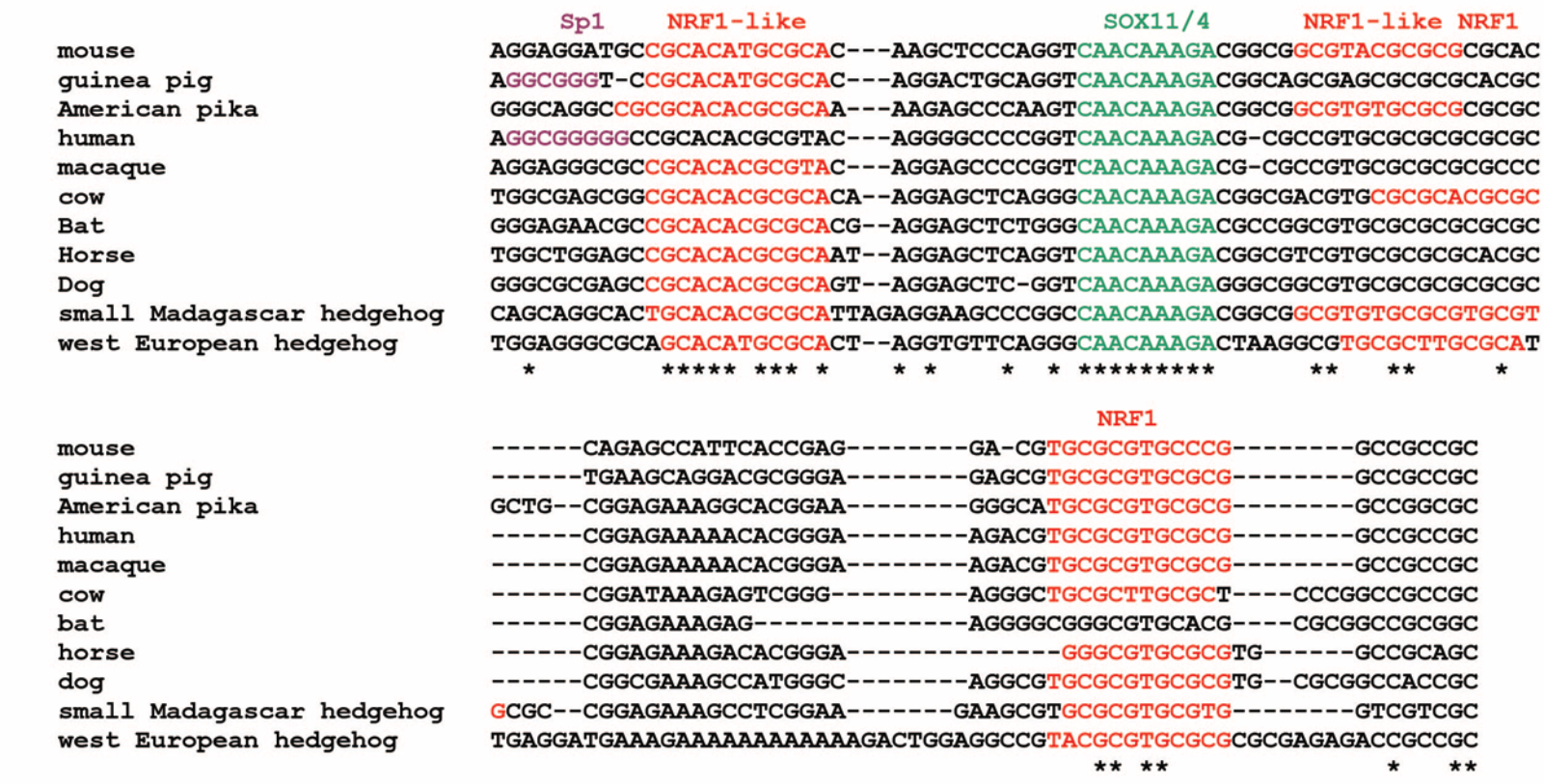


Figure 4.2. (a) (continued below)

(b) CLUSTAL 2.0.8 multiple sequence alignment for the *SPG5* (*CYP7B1*) exon 1-intron 1 region

```
                    NRF1              Exon 1  Intron 1                                                    NRF1
armadillo           CCTGTGCCTGCGCGCCCGCAGGACCAGGTAAGCGCC--GGCGGCCCGAGGGCG--GCCCCGTAG------CCGGGCCACGCATGCGCCCTCGC-CCC-GCGGACCGCGCGCGCGACCG
cow                 CTTGTGCCTGAATGCGCGGCGCACCAGGTAAGCGCCCCGACGACCCCGCGCCA--GCCC------------GGGCCACGCATGCGTCCTGGG-CCC---AGACCGCCCTCCATCC
horse               CCTGTGTCTGCGCGCCCAGCGCACCAGGTAAGCACCCCAGCCGCCTCGCCGCGAGGCGCAGCGGA-----CCGGGCCACGCATGCGCCCTGGG-CCCTGCGGACCGCGCGCGCGACCC
human               CCTCTGCTTGCTTGTCCGGCGCACCAGGTAAGCGCCTCGGCCGCC------------------------AGGCCACGCATGCGCCCTGGG-CCCCGCGGGCCGCGCGCGACCC
American pika       CCTGTGCCTGTGTGGCCGGCGCACCAGGTAAGCGCCCCGCCGCCC--------------------------GGACACGCATGCGCCCTGGG-CCCCGCCGCC---GTGCGACCC
dog                 CCTGTGCCTGCGCGCCCGGCGCACCAGGTAAGCGCCCCCGCGGCCTCGCCGCGCGGCCGGGCTC------CCGGGCCACGCATGCGCCCTGGT-CCCCGCGGGCCGAGCGCGACCC
cat                 CTTGTGCCTGACCTCCAGGCGCACCAGGTAAGCGCCCCCGCGACCTCACCGCGCGGCCGGGCA-----GCCAGGGCCACGCATGCGCTCTGGG-CCCCGCGGACCGCGCGCGACCC
European shrew      CCAGTGCCTGCGCTCCCGGCGCACCAGGTAAGTTTCCCCGCGGCCCCGCGCGTCGGCCGCGCGCCACGGCCCGGGCCACGCATGCGCTCTGGG-CGTCCCCGTCGGCTCGCGACCT
tree shrew          GCTGTGCCTGAGCTCCAGGCGCACCAGGTAAGCGCCCAGGCCGCC--------------------------GGGGCCACGCATGCGCTCTGGG-CCCTGCGGGCCGCGCGCGACCC
rabbit              CCTGTGCCTGCGCGGCCGGCGCACCAGGTAAGCGCCCCTGCCGCCGC------------------------GGCCACGCATGCGTCCTGGG-CCCCGCGGCC---GCGCGACCC
lemur               CCTGTGCCTGCGCGCACGGCGCACAAGGTAAGCGCCCCGACCGCCG-------------------------GGCCACGCATGCGCCCTGAAACCCCGGGGCCCGCGCGCGACCC
squirrel            CCTGTGCCTGCGCTCCAGGCGCATCAGGTAAGCGCCCGGGCCTTTG-------------------------GGGACACGCATGCGCCTTGGGCCCCCGCGGCCCGCGCGCGACCC
guinea pig          CCTGTGCCTGCGCGCCCGGCGCTCCAGGTAA---CCCCGGCGGGC--------------------------CCACGCATGCGCCCTGG---CCCGCGGCC---GCGCGCCCC
mouse               CCTGTTCCTCCTTACCCGGCGCACCAGGTAAGCGCCT-GGCT-----------------------------GGCCACGCATGCGT---GGG--ATCGCGGCC---ACGCGACCT
rat                 CCTGTTCCTCCTCACCCGGCGCACAAGGTAAGCGCCTAGGCT-----------------------------GGCCACGCATGCGT---GGG--ATCGCGGCC---ACGTAACCT
                        *   *           *     *****    *    *                                  **********                 *         *
```

(c) CLUSTAL 2.0.8 multiple sequence alignment for the *SPG6* (*NIPA1*) promoter

```
                                  NRF1    NRF1          Sp1         Sp1
human                   GGGGC---GCGGCGCGCAGGCGCAGGCTCGGAGGGC-GGGCGCGGGCG------------GAATGGGGACTGCAGCTGCGGCAGCG
baboon                  CGGGC---GCGGCGCGCAGGCGCAGGCTCGGAGGGC-GGGCGCGGGCG------------GAATGGGGACTGCAGCTGCGGCAGCA
rhesus monkey           CGGGC---GCGGCGCGCAGGCGCAGGCTCGGAGGGC-GGGCGCGGGCG------------GAATGGGGACTGCAGCTGCGGCAGCG
rabbit                  CGGGC---GCGGCGCGCAGGCGCAGGCACGGAGGGC-GGGCGCGGGCG------------GAATGGGGACTGCAGCGGCGGCAGCG
dog                     CCGGC---TCGGCGCGCAGGCGCAGGCGCGGAGGGC-GGGCGCGGGCGCGGGCGCGGGCGGAATGGGGACTGCAGCGGCGGCGGCG
west European hedgehog  CGGGC--GC-GGTGCGCAGGCGCGGA---GGGCGGC--GGCGCGGGCG------------GAATGGGGACTGCAGCGGGGGCAGCG
horse                   CGGGC--GCTGGCGTGCAGGCGCAGACAAGGACGGGAGGGCGCGGGCC-----------AGAATGGGGTCTGCAGCGGCGGCAGCG
cow                     CGGGCTGGCGAGCGCGCAGGCGCAGA---GGGCGGG----CACGGGCG------------GAATGGGGACTGCGGCTGCTGCAGCG
mouse                   GGGGCGCG-GCGCGCGCACGCGCAGC---GGAGGGC--GGCGCGGGCG------------GCATGGGGACTGCGGCGGCGGCTGCG
guinea pig              CGG-CGCA-GAG-GCGCAGGCGCGAGGGCG----------CGCGGGCG------------GCATGGGGACTGCAGCGGCTGCGGCG
European shrew          CGGGCT-TCTT-TTCGCAGGCGCAGGCGCGGGCCGC-----GCGGGCG------------GGATGGGGACTGCGGCGGCGGCAGCG
Monodelphis             CGTGAT--CCCAGCGGTGTGCGCAGGCGCGGGAACCCGGGCCGCTz------AGGGAGGAAGATGATGACTGCTACTGCAGCAGCC
platypus                CGTC-TCGGAGGGCCTTGCGCGCATGCGCCGG----CGGAGGAGGZ--------GGAGCGGGATGGCTGCAGTGGCCGGGGAAGGG
                                        ****                                          ***    * *   * *  *  *
```

where z (36-nt) and Z (28-nt) are insertions in *M. domesticus* and platypus, respectively:

```
Monodelphis     TTCGGTAGCAAGTAGGGAGGAAGCTTCGGCTCTGCT
platypus        TTCGGGAGC-----GGGAG-CCGGAGCGG--GAGCC
                ***** ***     *****   *   ***    **
```

Figure 4.2. (b), (c) (continued below)

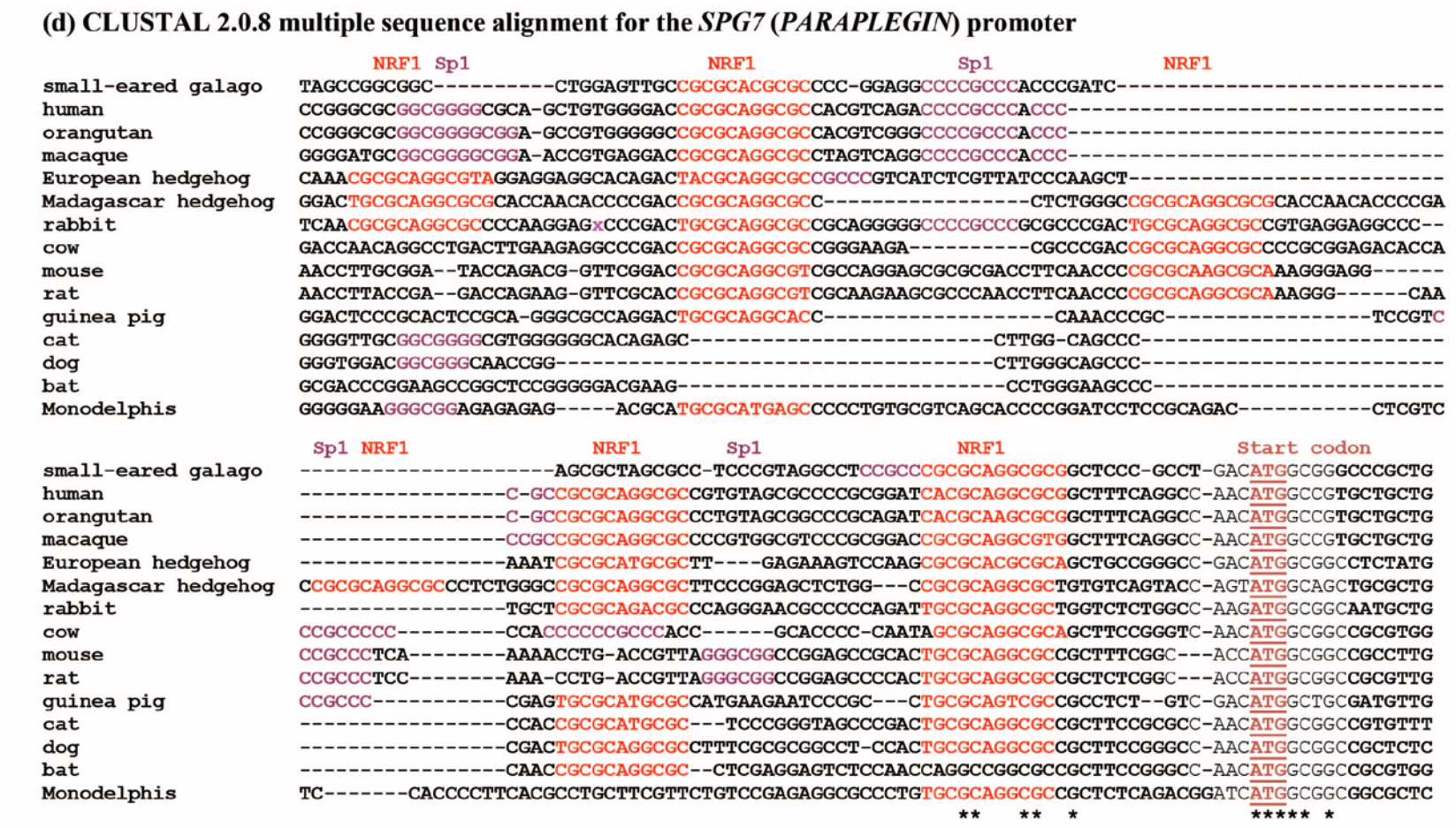


Figure 4.2. Evolutionary conserved NRF1 binding motifs in 5' regulatory regions of HSP loci. Multi-sequence alignments were generated using Clustal W with manual adjustments, and representative examples are shown (* = conserved nucleotides): (a) *SPG4*, (b) *SPG5*, (c) *SPG6*, and (d) *SPG7*. In (c), z (36-nt) and Z (28-nt) are species-specific insertions in *M. domestica* and platypus, respectively; in (d), x is a 31-nt insertion in the rabbit.

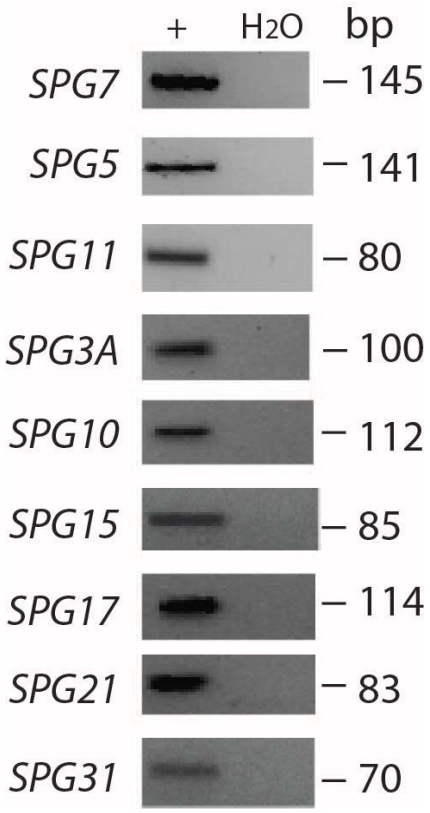


Figure 4.3. HSP gene expression studies in human SK-N-SH cells. bp: base pair. H_2O is PCR control.

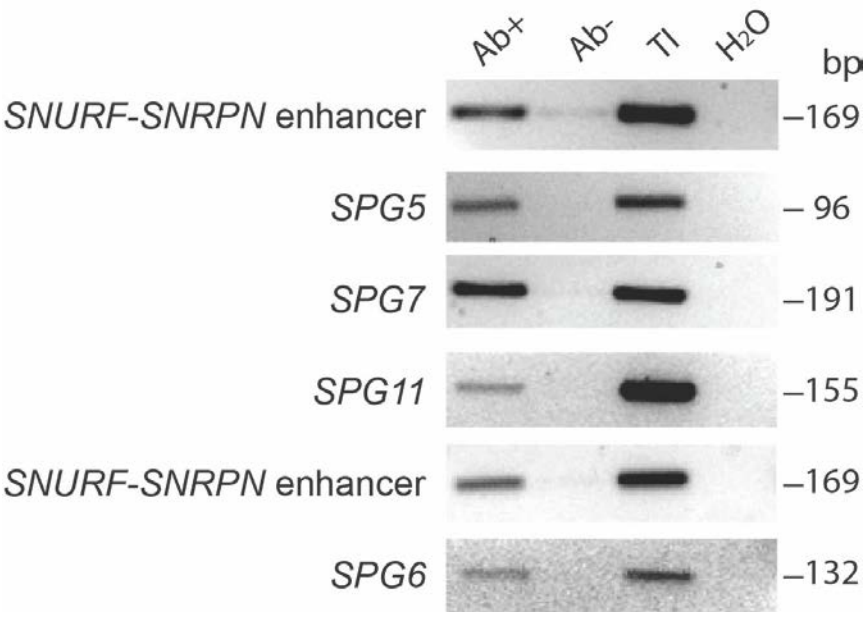


Figure 4.4. NRF1 binds at HSP gene promoters in human SK-N-SH cells by ChIP. Antibody positive is shown as Ab+, controls are no antibody (Ab-), total input (TI) DNA is for PCR positive control, H_2O is PCR negative control, and the positive ChIP control is the *SNURF-SNRPN* enhancer (Rodriguez-Jato *et al.*, 2005).

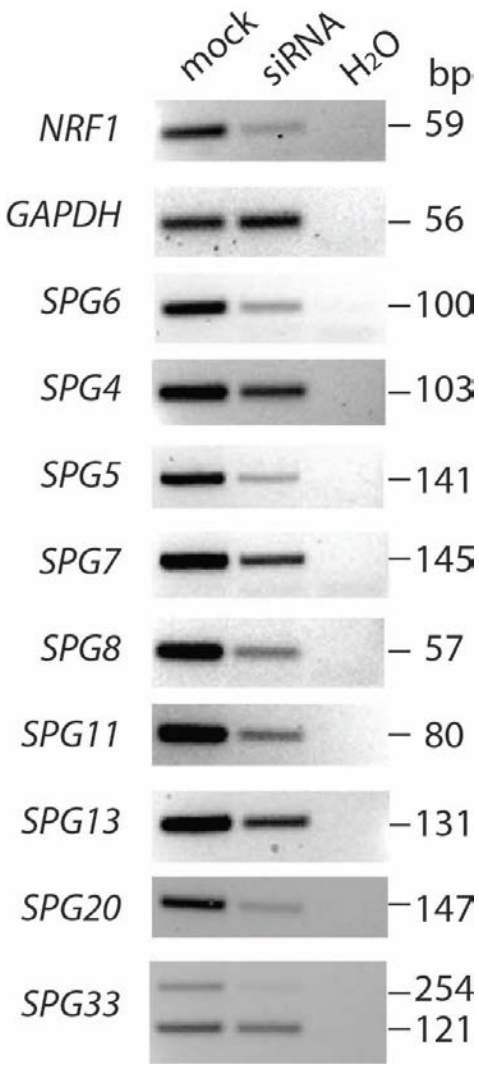


Figure 4.5. siRNA targeting NRF1 mRNA knocks down HSP gene expression. SK-N-SH cells were transfected with pSUPER-*NRF1* siRNA or mock transfected as a control, with qualitative analysis of gene expression including a negative control (*GAPDH*). The *GAPDH* mRNA level is unaffected by *NRF1* siRNA, whereas the mRNA levels for *NRF1* and all shown HSP genes are reduced by the siRNA treatment (including two mRNA splice isoforms for *SPG33*).

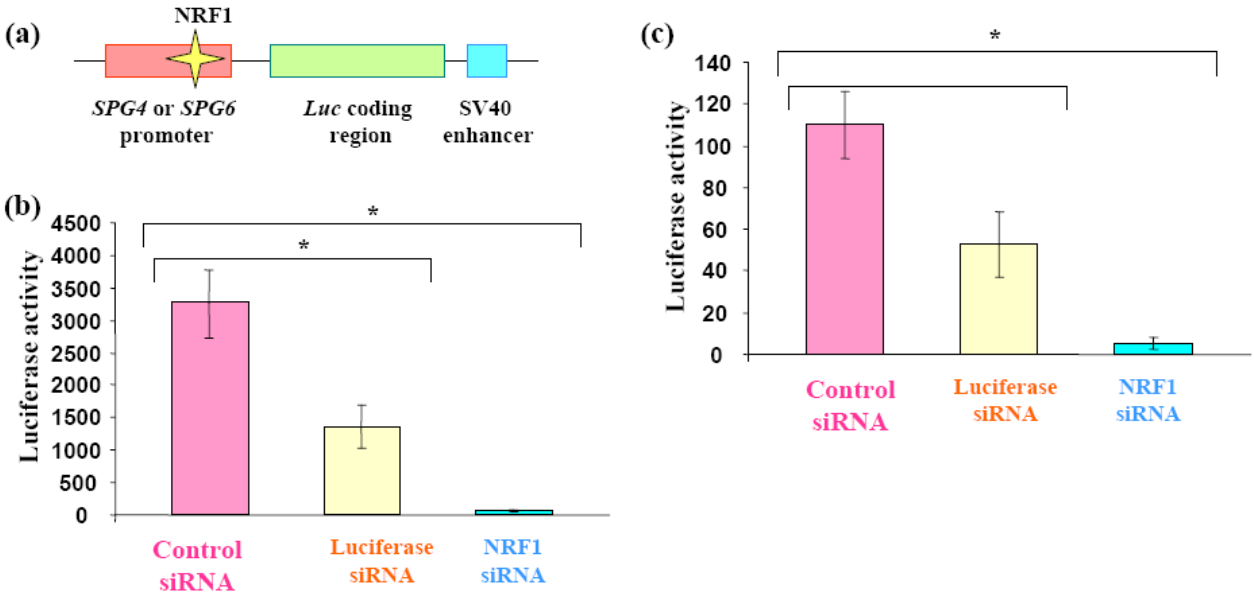


Figure 4.6. siRNA targeting the NRF1 mRNA ablates *SPG4* and *SPG6* promoter function. A pGL3e-SPGx-promoter [x = 4 or 6; constructs are shown in **(a)**] plasmid and a siRNA plasmid (either targeting an *Arl2* control, *Luc*, or *NRF1*) were co-transfected into SK-N-SH cells. Luciferase activities were measured for the **(b)** *SPG4* promoter and **(c)** *SPG6* promoter. *, $P < 0.05$, for the control siRNA versus test siRNA (targeting *Luc* or *NRF1*).

4.3.2 NRF1 autoregulates and enhances transcriptional activity

Since we found evolutionary conserved potential NRF1 binding sites in the 5' regulatory region of the gene, we then used molecular assays to confirm these findings. First, to test whether NRF1 binds this region, we used a ChIP assay and confirmed that NRF1 binds this site in the human SK-N-SH neuroblastoma cell line [**Figure 4.7(a)**], in the mouse Neuro2A neuroblastoma cell line (data not shown), and to the conserved, homologous region in a marsupial fibroblast cell

line [**Figure 4.7(b)**]. As shown by phylogenetically conserved motifs (**Figure 4.1**), YY1 is another conserved binding motif we found in the promoter of NRF1. Similarly, we used ChIP to confirm that YY1 binds to the *NRF1* promoter in SK-N-SH cells [**Figure 4.7(c)**] and Neuro2A cells (data not shown).

Next, we used luciferase reporter assays to test the degree of effects that each TF has on the regulation of NRF1. We cloned a minimal *NRF1* promoter-exon 1 sequence, including one Sp1 site, two NRF1 sites and one YY1 site, into a pGL3basic luciferase vector and generated mutations of 1) each and 2) both NRF1 sites, as well as 3) the YY1 site (**Figure 4.8**). Then, we co-transfected each pGL3basic derived construct with a *Renilla* luciferase vector as an internal control into a human peripheral neuronal precursor cell line, Flp-In 293 cells. As shown in **Figure 4.8**, the WT *NRF1* promoter-exon 1 promoter significantly increased luciferase activity by 87-fold compared to the empty vector ($P<0.001$), indicating a functional promoter construct. The promoter activities of all mutant constructs had a dramatic decrease in luciferase activity when compared to the WT construct (**Figure 4.8**). Specifically, when the YY1 site was mutated, the luciferase activity decreased by 67% or 29-fold ($P<0.05$). Mutation of either 5'- or 3'-NRF1 site decreased luciferase activity to 58% or 51-fold ($P<0.05$) and to 55% or 48-fold ($P<0.05$), respectively; whereas mutations of both NRF1 sites decreased luciferase activity to 44% or 39-fold ($P<0.05$). These results indicate that both NRF1 sites have a significant effect on regulating its own promoter to increase transcriptional activity. Nevertheless, the two NRF1 sites do not synergize in transcriptional activity, suggesting that only one NRF1 homodimer binds this region in its own promoter. This is probably due to the spacing between the two NRF1 sites in this region that is too small (4 nucleotides apart, **Figure 4.1**) for two NRF1 homodimers to bind

efficiently, since a minimum of 8 nucleotides between two NRF1 sites are needed (Virbasius & Scarpulla, 1994).

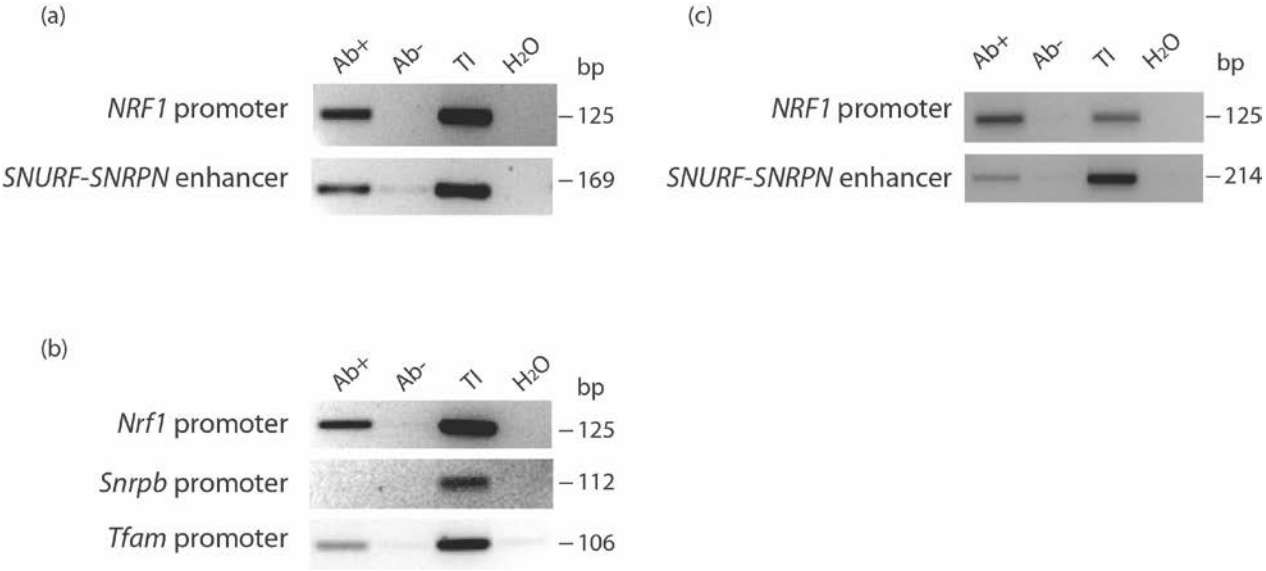


Figure 4.7. ChIP studies of the *NRF1* promoter-exon 1 region. (a) NRF1 ChIP in SK-N-SH cells. **(b)** NRF1 ChIP in marsupial cells. **(c)** YY1 ChIP in SK-N-SH cells. Antibody positive is Ab+, controls are no antibody (Ab), total input (TI) DNA is for PCR positive control, H_2O is PCR negative control, and the positive ChIP control is the *SNURF-SNRPN* enhancer (Rodriguez-Jato *et al.*, 2005).

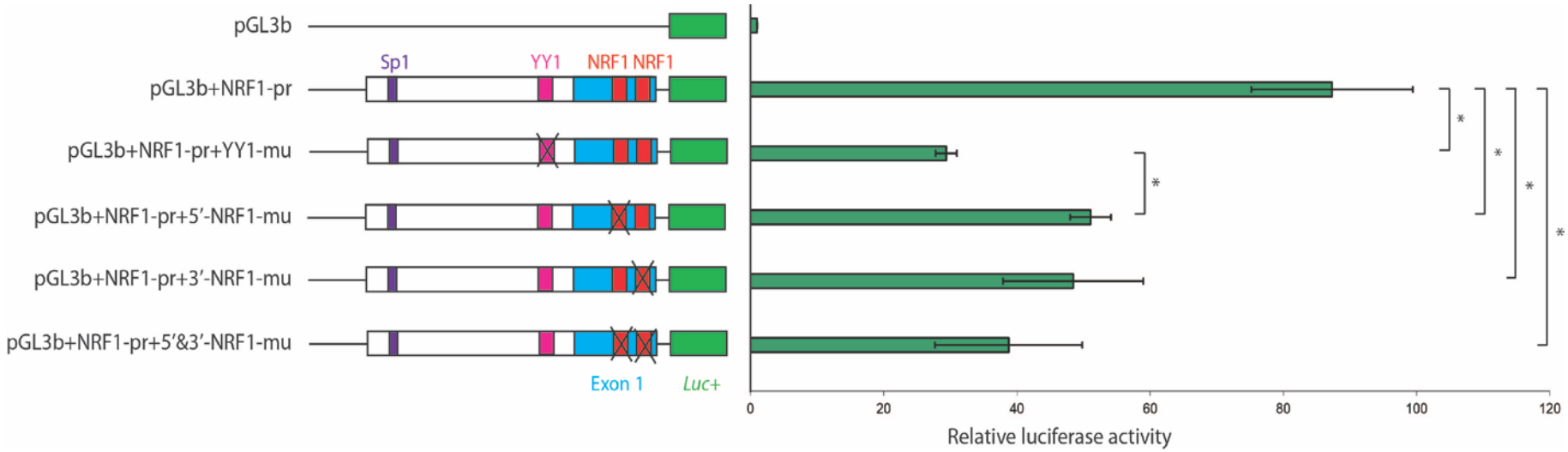


Figure 4.8. Luciferase reporter assays for *NRF1* promoter activity. *NRF1* promoter (pr) luciferase constructs (left) showing TF binding sites (Sp1, YY1, NRF1) and mutations (mu; X) in TF sites. Luciferase reporters assays (right). *, $P < 0.05$

4.3.3 Relationship between number of NRF1 sites and promoter activity

Luciferase reporter vectors with 10, 8, 6, 4, 2, or 1 copies of NRF1 binding sites were cloned into a modified pGL3basic vector to test how the number of NRF1 sites affect the level of transcriptional activity. The results indicated that these artificial NRF1-only sequences provided significant promoter function, and that luciferase activity increased as the number of NRF1 sites was increased [**Figure 4.9(a)**]. However, the relationship between the number of NRF1 sites in the promoter and effect on activating transcription was not purely linear [**Figure 4.9(a)**]. In this system, two NRF1 sites had 6-fold increased luciferase activity compared to one NRF1 site, while doubling to four NRF1 sites increased activity a further 14-fold (80-fold over a single NRF1 site). For unknown reasons, increasing to six or eight NRF1 sites had only no or small effect on luciferase activity, respectively, whereas ten NRF1 sites had a 2-fold further increase in promoter activity compared to eight NRF1 sites and for a total of 245-fold that of a single NRF1 site [**Figure 4.9(a)**]. In testing the correlation between the number of NRF1 sites versus the level of transcriptional activity, there was a strong correlation [correlation=0.94, **Figure 4.9(b)**]. In Excel, such data was plotted and fitted the best to a polynomial regression with order of 2 [R^2=0.9224, **Figure 4.9(b)**]. Therefore, I conclude that the number of NRF1 sites has positive effect in activating transcriptional activity of a promoter in the luciferase reporter system.

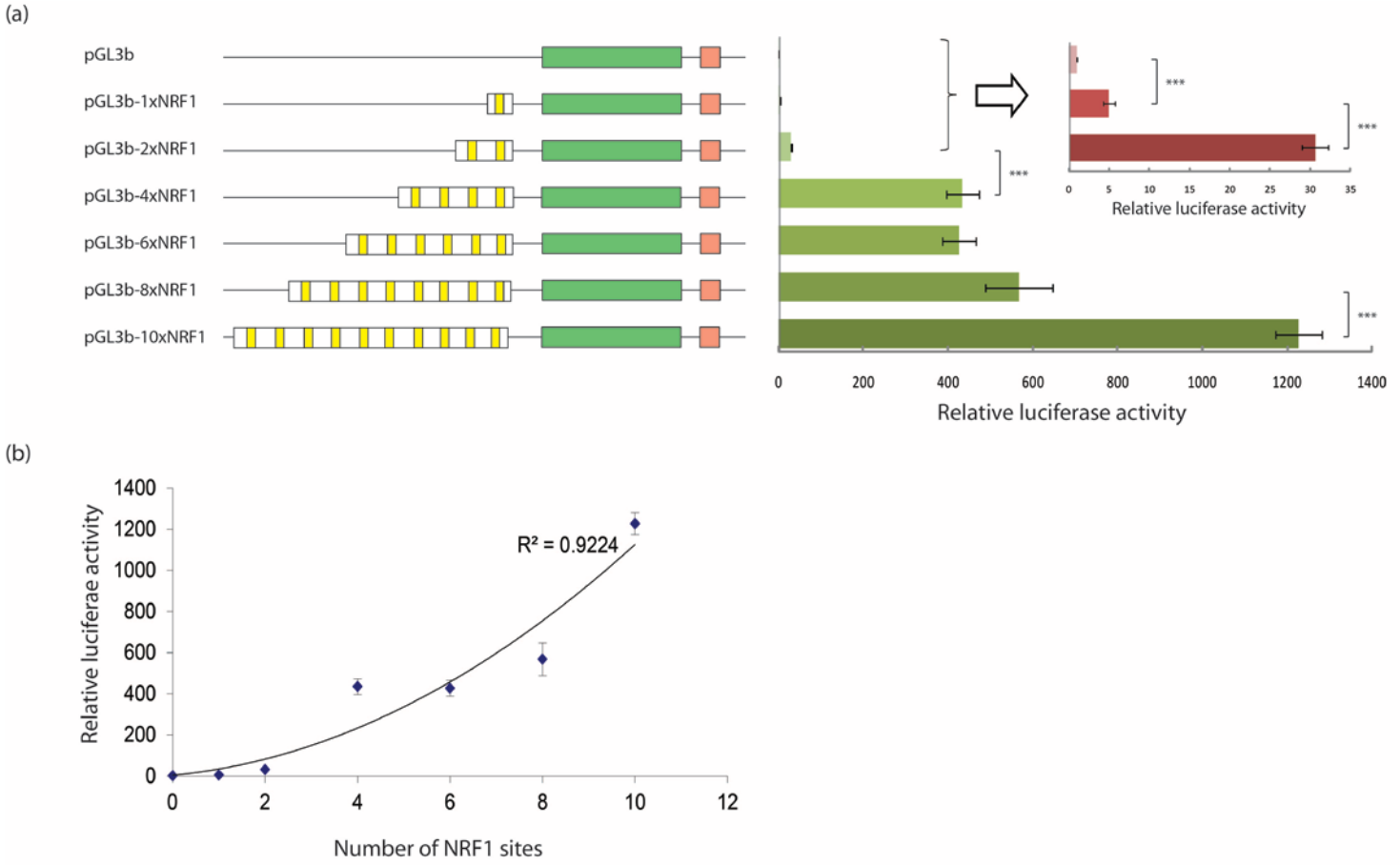


Figure 4.9. Luciferase reporter assays for NRF1 binding site tandem arrays. (a) NRF1 tandem array luciferase constructs (left). Luciferase reporters assays (right). *, $P < 0.05$; **, $P < 0.001$; *, $P < 0.0001$. (b) Correlation between relative luciferase activity versus the number of NRF1 sites.

4.4 DISCUSSION

4.4.1 NRF1 regulates spastic paraplegia genes *in trans*

By bioinformatics analysis and molecular assays, we have shown that NRF1 regulates many HSP genes. Our findings suggest that NRF1 may contribute significantly to gene regulation in the cortical neurons that extend corticospinal axons which degenerate in HSP. As would be expected, in some of these HSP gene promoters, there are also other conserved TF binding sites. For example, there is a highly conserved site for SOX4 and SOX11 in the *SPG4* promoter [**Figure 4.2(a)**]. Mutation of this site significantly reduced luciferase activity while activity of the *SPG4* promoter was significantly increased with SOX11 overexpression (B. Henson, R.D. Nicholls, personal communication).

As well as transcriptional regulation, it has become increasingly clear that a major additional regulatory mechanism exists in cells with modulation of mRNA stability and/or the level of translation of an mRNA by a novel functional class of very small RNA molecules termed microRNAs (miRNAs; Rivera & Bennett, 2010). Typically, mature, processed miRNAs are single-stranded RNAs 20-25 nucleotides in length (Chi *et al*., 2009). Mechanistically, miRNAs base-pair with their RNA targets, beginning with a 6-8 nucleotide "seed" sequence localized at the 5' end of a miRNA (Chi *et al*., 2009). The "seed" provides sufficient specificity for each miRNA to potentially regulate several hundred mRNAs with a range of less than 100 to over 1000 targets (Chi *et al*., 2009). Other members in our laboratory have shown that the miR-183/96/182 family miRNAs target sites in one-third of HSP mRNAs (B. Henson, R.D. Nicholls, personal communication). This provides further insight into the GRNs for HSP genes. An understanding of transcriptional plus miRNA regulatory signatures may help to identify and rank

the top candidate genes within large chromosomal domains linked to more than 20 uncloned HSP chromosomal regions (Blackstone *et al*., 2011).

4.4.2 NRF1 autoregulation

By phylogenetic analysis, we showed that NRF1 and YY1 binding sites in the *NRF1* promoter are within a functionally selected region over the past ~450 million years (**Figure 4.1**). These NRF1 and YY1 sites are also conserved in *Ciona* species (data not shown), among the most primitive vertebrates; further attesting to the importance of these regulatory features. Our molecular results demonstrated that NRF1 acts by a feed-forward mechanism to regulate its own transcriptional activity in a positive way. Based on the results from luciferase reporter assays, YY1 is also a transcriptional activator for the *NRF1* promoter. Since Sp1 is a common transcriptional activator in most mammalian promoters of the CpG-rich class (Pugh & Tjian, 1990), we hypothesize that there must be a negative mechanism of TF regulation associated with the *NRF1* promoter to repress its gene expression. Within intron 1 of *NRF1*, a conserved DNA element corresponds to a putative C/EBP (CCAAT/enhancer-binding protein) site (**Figure 4.1**). It was reported that C/EBP can either be a transcriptional activator (Landschulz *et al*., 1988) or repressor (Pei & Shih, 1990). Therefore, the next step in better understanding the transcriptional regulation of the *NRF1* gene is to test if C/EBP is the TF that binds to this site and whether this site positively or negatively regulates *NRF1* transcription. To further assess the complex question of how NRF1 regulates itself *in vivo*, it will be interesting to overexpress NRF1 protein in a cell line model and investigate how the increase of NRF1 protein level affects the transcription of *NRF1* mRNA to maintain homeostasis of the NRF1 protein levels.

4.4.3 Relationship between number of NRF1 sites and promoter activity

Here, I reported that number of NRF1 sites in an artificial promoter has a positive role in regulating transcriptional activity. Indeed, similar natural clusters of NRF1 sites occur in transcriptional regulatory sequences. For example, *SPG7* promoter has 1 (bat) to 6 (small Madagascar hedgehog) NRF1 sites [**Figure 4.2(d)**], *SPG4* promoter has up to 3 NRF1 sites [**Figure 4.2(a)**], "NRF1 cluster" enhancer in the PWS domain has 4 sites (**Figure 2.2**), and 5' regulatory regions of other candidate genes have various number of NRF1 sites, e.g., *EIF5A* (eukaryotic translation initiation factor 5A, 7 NRF1 sites), *MAP2K7* (mitogen-activated protein kinase kinase 7, 4 NRF1 sites), etc (data not shown).

Numerous studies have found that spacing between TF binding sites is also a critical determinant of gene expression (Vilen *et al*., 1991; Nolte *et al*., 2006; Cheung *et al*., 2007; Larsson *et al*., 2007). Therefore, it provides another direction in exploring the structure and affinity of NRF1 binding. The artificial NRF1 site arrays discussed above or the "NRF1 cluster" enhancer element from the PWS domain would provide suitable reagents to alter the spacing of the NRF1 sites to assess the effect on promoter or enhancer activity in *in vitro* luciferase studies. Similarly, the parameters of match to consensus NRF1 recognition sequence could also be studied in this assay system, since the structure of NRF1 has not been determined, such studies could provide insights into the properties of DNA binding by NRF1.

This study has served as a prelude to a future goal of determining the structure of NRF1, the mode of binding to target sites, and changes in affinity for different targets. In the long term, such studies may help to develop small molecule regulators of NRF1 function as drugs in diseases involving NRF1 deregulation, including diabetes, obesity, cancer and neurological diseases.

5.0 CONCLUSIONS AND FUTURE STUDIES

5.1 CONCLUSIONS

A GRN is a central processing unit in a living cell. GRN are composed of a collection of DNA segments which interact with each other by RNAs or proteins through intracellular and/or extracellular signals, thereby governing the rates at which genes in the network are transcribed into mRNA (Macneil & Walhout, 2011). A major class of regulators in a GRN is TF (Macneil & Walhout, 2011). The binding of TFs to specific DNA elements provides a major mechanism in the fundamental basis of a GRN (MacQuarrie *et al*., 2011). Other components in a GRN include RNA regulators, e.g., miRNA and lincRNA (long intergenic noncoding RNA, Wang *et al*., 2011), and chromatin factors, such as histone modifications (Macneil & Walhout, 2011). For over half a century, studies have been focused on TF regulation in order to study GRN (Chen & Rajewsky, 2007). The development of computational analysis in biological fields enables a preliminary scan for TF binding sites in the human genome, for which the biological importance can initially be tested by molecular phylogenetic analysis due to the wealth of animal whole genome sequences. The advent of new molecular techniques, such as ChIP–chip and ChIP-seq, allows more efficient direct experimental confirmation of bioinformatics results (Pareek *et al*., 2011). Other technologies, such as siRNA knockdown of an mRNA encoding a TF and hence of protein levels, or TF overexpression, followed by whole genome RNA analyses by expression

microarrays or RNA-seq (a next generation sequencing approach) also provide confirmation by dysregulation of expression of target genes for a specific TF. High throughput technologies in experimental model systems are now allowing assessment of more than one factor at a time in system approaches (Pareek *et al*., 2011).

In my research projects, I tried to make good use of both bioinformatics analysis and experimental techniques to elucidate the mechanisms and functional roles of NRF1 binding sites in the human genome. In particular, I have focused on NRF1 regulation in the 2 Mb PWS domain and the circadian system. In addition, I started to explore the function of NRF1 in regulating HSP genes and its own autoregulation. NRF1 binding sites were found in 5-6% of the promoters in the human genome by bioinformatics searches and ChIP-chip studies (Cam *et al*., 2004; FitzGerald *et al.*, 2004; Xi *et al*., 2007). It is known that many nuclear genes encoding mitochondrial products are regulated by NRF1 (Scarpulla, 2008). In our laboratory, bioinformatics analysis and molecular studies showed that NRF1 is overrepresented in the three systems we studied. NRF regulates 83% of the genes in the 2 Mb PWS region, 56% in circadian regulatory genes, and 45% in HSP genes (**Figure 5.1**). My studies have provided important evidence on NRF1 binding elements and initial studies towards identifying GRNs that involve NRF1.

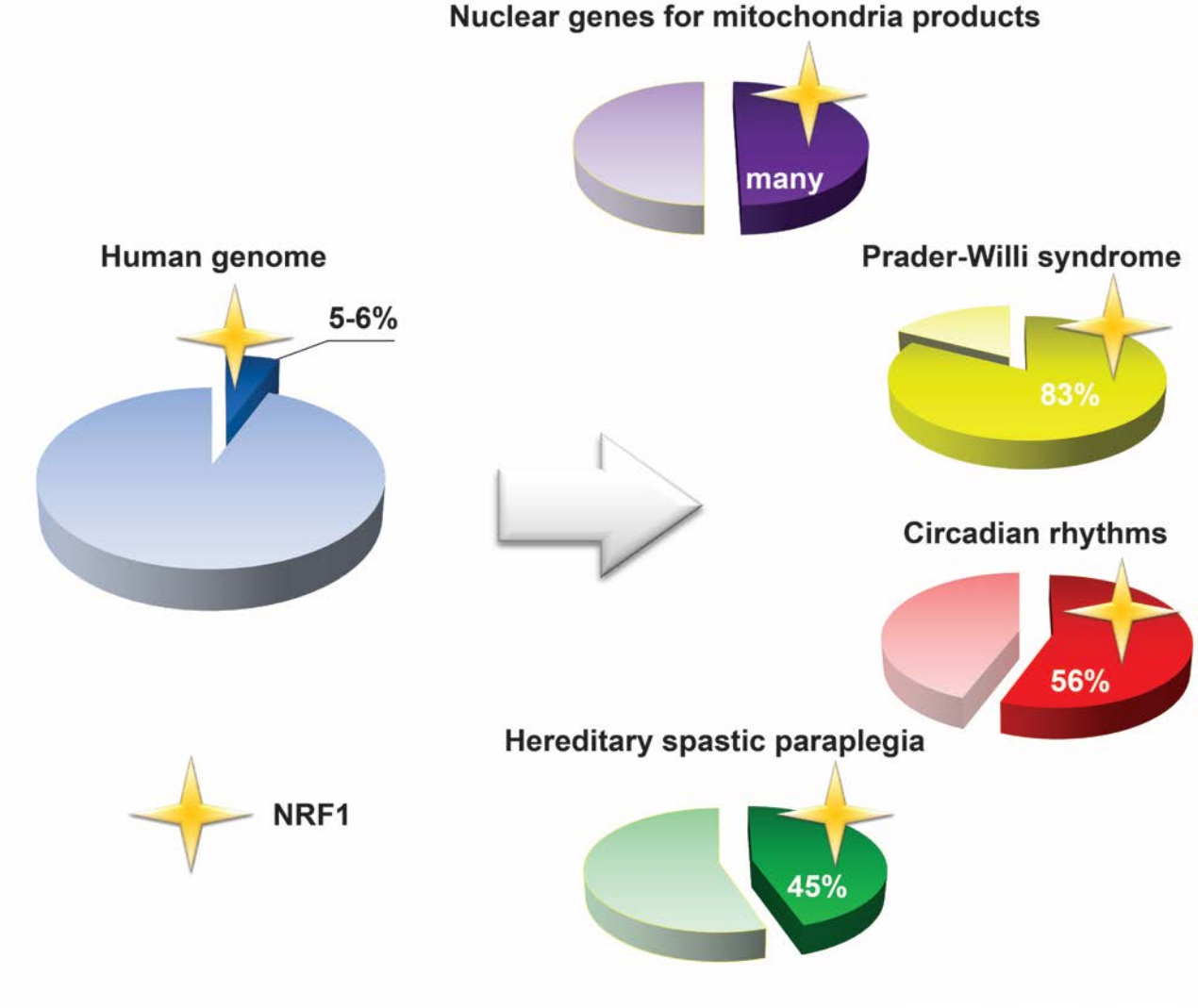


Figure 5.1. Systems with enrichment of transcriptional regulation by NRF1 in the human genome.

5.2 FUTURE STUDIES

For future work, we should not only focus on the study of specific TF binding sites such as NRF1, but also on *cis*-regulatory modules, that include binding sites for NRF1 and other TFs with which it may co-regulate different classes of genes. In **Chapter 2**, an original goal was to use somatic cell hybrids for a gene targeting experiment in order to study the mechanism of the "NRF1 cluster", a putative *cis*-regulatory module with enhancer function, on long-range regulation of the 2 Mb PWS-imprinted domain. Although my studies in somatic hybrid cells showed that none of these cell lines were suitable for gene targeting of the "NRF1 cluster" due to a lack of active chromatin at this site in these cells, the somatic cell hybrids studies nevertheless provided gene expression and chromatin configuration profiles of NRF1 binding and histone methylation for understanding gene regulation over the 2 Mb imprinted PWS domain. Optimally, the *cis*-regulatory role(s) of the "NRF1 cluster" can be investigated by gene targeting in mouse embryonic stem cells and subsequent analysis in knockout mice, to determine the extent to which this element is required for active chromatin an how many of the genes it regulates in a neural-specific manner within the PWS-orthologous domain in the mouse.

In the study of circadian rhythms, my work indicates that NRF1 acts as a potential master regulator of this complex system with targets in both the positive and negative feedback regulatory loops. It will be crucial to study how NRF1 interacts with CLOCK/BMAL1 heterodimers in a molecular complex and regulates the expression of other circadian regulatory genes. In addition, studies of activity patterns, sleep, and metabolism in *Nrf1* +/- heterozygous mice compared to WT controls will be of significant interest. Identification of overrepresented motifs in sequences associated with NRF1-regulated elements in circadian regulatory genes can help to identify additional potential TFs that may interact and/or function with NRF1 in the

circadian system. Other future studies would examine the role of NRF1 in SCN neurons and neuronal input and/or output pathways to the central circadian regulator.

My studies of NRF1 binding sites in HSP genes have provided substantial data for our laboratory to continue studies of the function of NRF1 in this complicated neural system. Recent studies have confirmed that another TF, SOX11, regulates *SPG4* (see **Figure 4.2**) along with NRF1 (B.J. Henson, R.D. Nicholls, unpublished data). Further, our laboratory has also identified overrepresented miRNA target sites in HSP mRNAs, predicting that the miR-96/-182 family miRNAs target nine HSP loci, an 8.5- to 11.5-fold increase over expectation (B.J. Henson, R.D. Nicholls, unpublished data). To date, our laboratory has confirmed miR-96/-182 regulation of *SPG4*, *SPG6*, *SPG1*, and of *NRF1*, with dynamic regulatory effects on mRNA and translation levels depending on cellular conditions. This work has therefore identified major TF and miRNA regulators within GRN important for neuronal function and maintenance of motor cortex axons that extend along the corticospinal tracts that degenerate in HSP. Future studies will identify additional transcriptional and miRNA regulatory elements for known HSP genes, and our laboratory plans to use the NRF1 + miR-96/-182 regulatory signature to identify and rank the top candidate genes within large chromosomal domains linked to uncloned HSP loci to aid identification of the disease genes. In the long term, this research program is identifying new functional elements for mutation studies in HSP, will provide a streamlined set of candidate genes for more rapid and cheaper identification of uncloned HSP genes, and may provide new targets for novel therapeutic approaches in neurodegenerative diseases.

Obesity and diabetes are prominent public health issues in the USA and worldwide, and are associated with cardiovascular and other diseases (Lowell & Shulman, 2005; Keller, 2006; Kouris-Blazos&Wahlqvist, 2007). It has been suggested that variation in genetic loci and

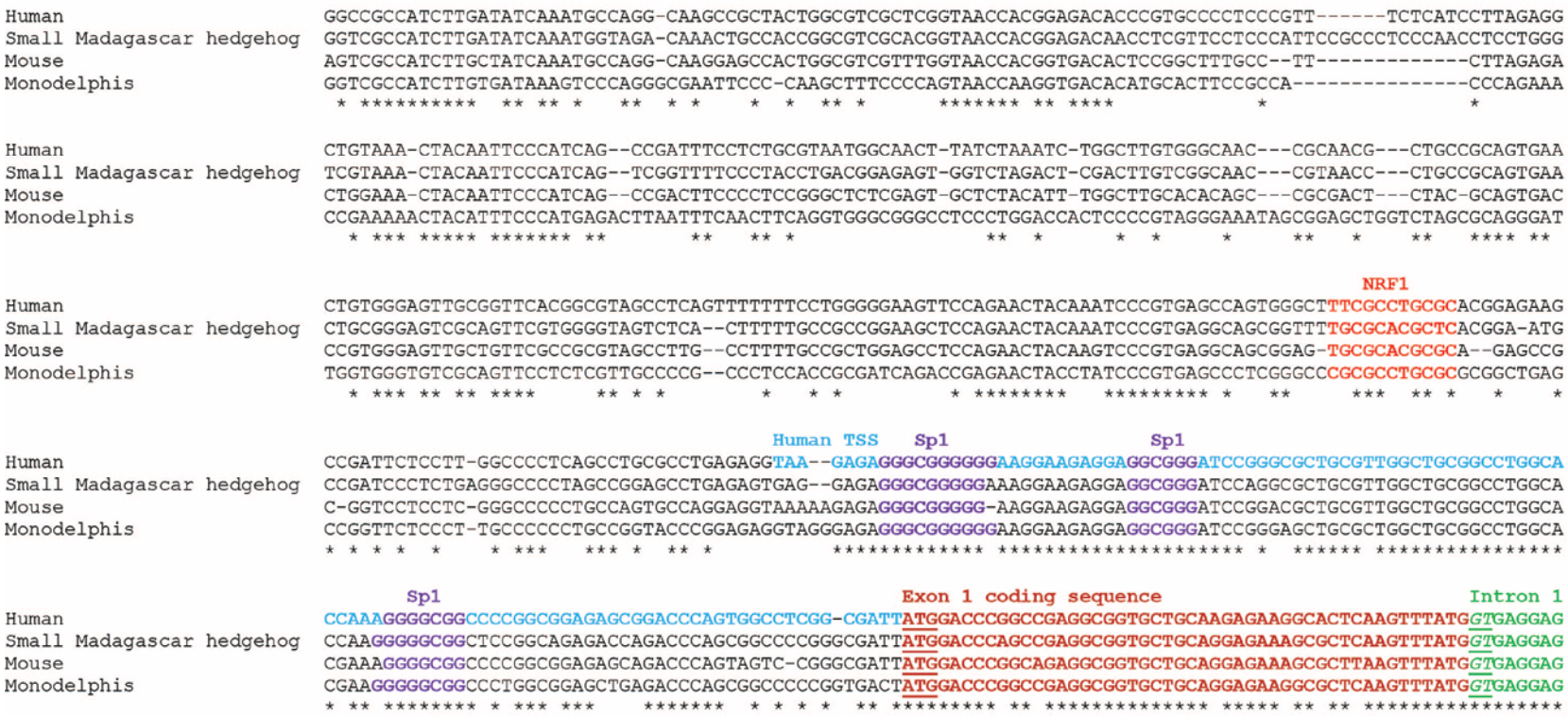
environmental factors have additive effects on metabolic and mitochondrial pathways in type II diabetes (Lowell & Shulman, 2005). Intriguingly, many genes encoding mitochondrial functions and regulators, including NRF1, have decreased expression in muscle from type II diabetes patients (Patti *et al*., 2003). A recent study has suggested genetic polymorphisms linked to *NRF1* could be a susceptibility factor for type II diabetes possibly by conferring abnormalities in triglyceride metabolism (Liu *et al*., 2008). Also, studies in our laboratory have found that NRF1 is a master regulator of gene expression in the PWS region (Stefan *et al*., in preparation), a neurobehavioral disorder associated with childhood onset of severe obesity, and that NRF1 regulates multiple genes involved in type II diabetes (M. Stefan, R.D. Nicholls, unpublished data). Furthermore, regulatory genes in circadian rhythms are also implicated in diabetes and obesity (Bass & Takahashi, 2010; Huang *et al*., 2011). Therefore, a better understanding of NRF1 target genes and pathways may provide important insights into the biological pathways and pathogenesis of diabetes, obesity, and other human disease not yet studied in detail (e.g., cancer), in addition to the roles that we have defined for NRF1 regulation in PWS, HSP, and circadian function.

APPENDIX

[MULTI-SEQUENCE ALIGNMENTS FOR 20 CIRCADIAN REGULATORY GENE PROMOTERS OR ENHANCERS]

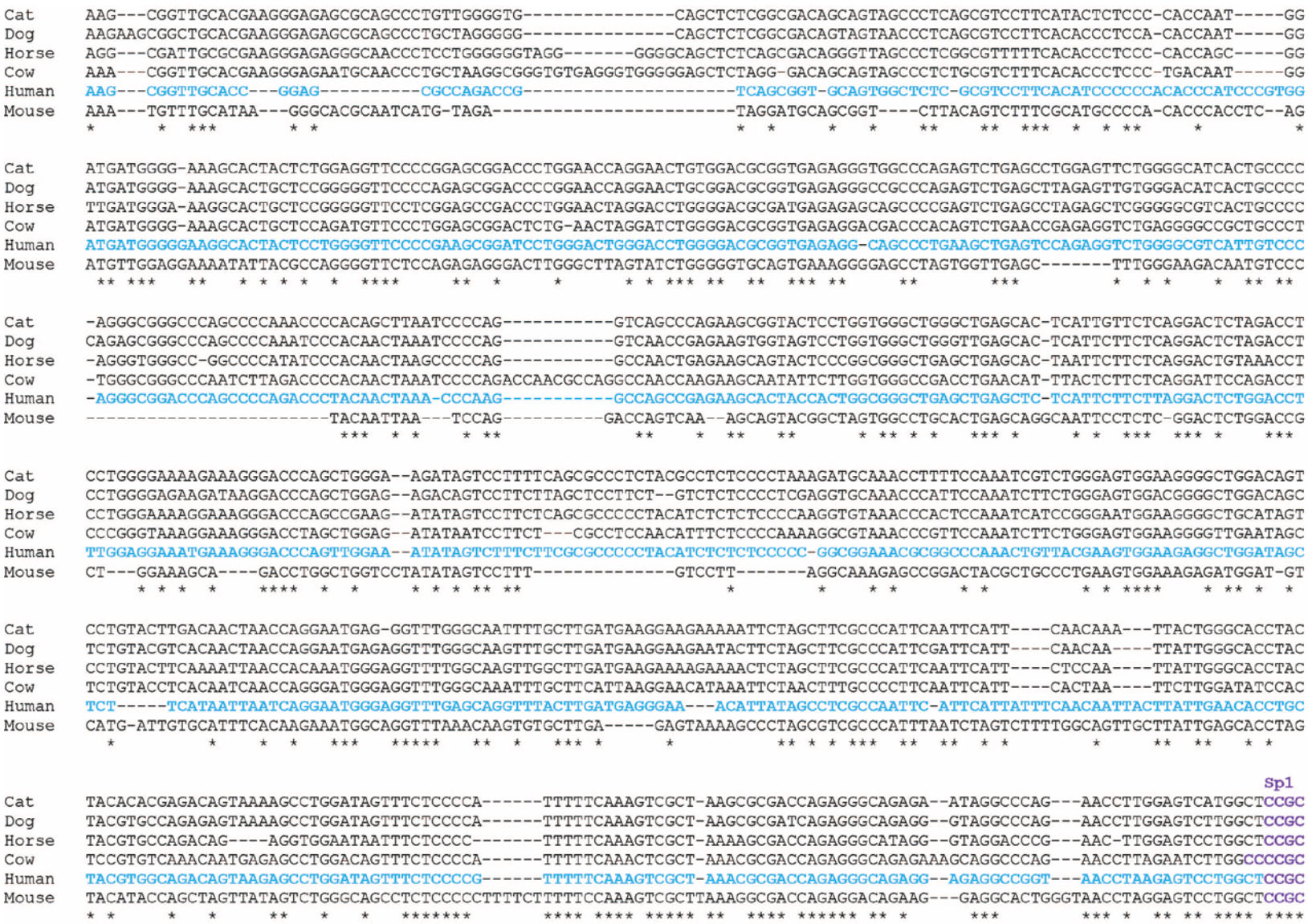
(a) *BTRC* promoter (ChIP); (b) *PPP5C* promoter (ChIP); (c) *FBXL15* promoter (ChIP); (d) *WDR5* promoter (ChIP); (e) *TIMELESS* intron 1 (ChIP); (f) *TEF* promoter (ChIP); (g) *HSF1* promoter (ChIP); (h) *EZH2* promoter (ChIP); (i) *CIPC* promoter (ChIP); (j) *CHORDC1* promoter (ChIP); (k) *ALAS1* promoter (ChIP, pub); (l) *FMR1* promoter (pub); (m) *FXR2* promoter (pub); (n) *CSNK1E* intron 1 (no exp); (o) *NR1D2* intron 1 (no exp); (p) *CREM* promoter (no exp); (q) *MYBBP1A* promoter (no exp); (r) *RAB3A* intron 1 (no exp); (s) *ATF5* intron 1 (no exp); and (t) *PROK2* promoter (no exp). Consensus sequences for CLOCK/BMAL1 (E-box), NRF1, NF-Y, RORE, DBP, and Sp1 motifs are highlighted in pink, red, green, orange, brown, and purple, respectively. ChIP indicates those *cis*-regulatory elements that we have shown bind NRF1 in SK-N-SH cells (see **Figure 3.2**). Previous publications (pub) have established that NRF1 regulates three of these promoters (for ALAS1, FMR1, and FXR2) but we present here expanded multisequence alignments. For seven genes, no experimental (no exp) data has been obtained, other than the phylogenetic conservation presented here.

(a) *BTRC* promoter (ChIP)



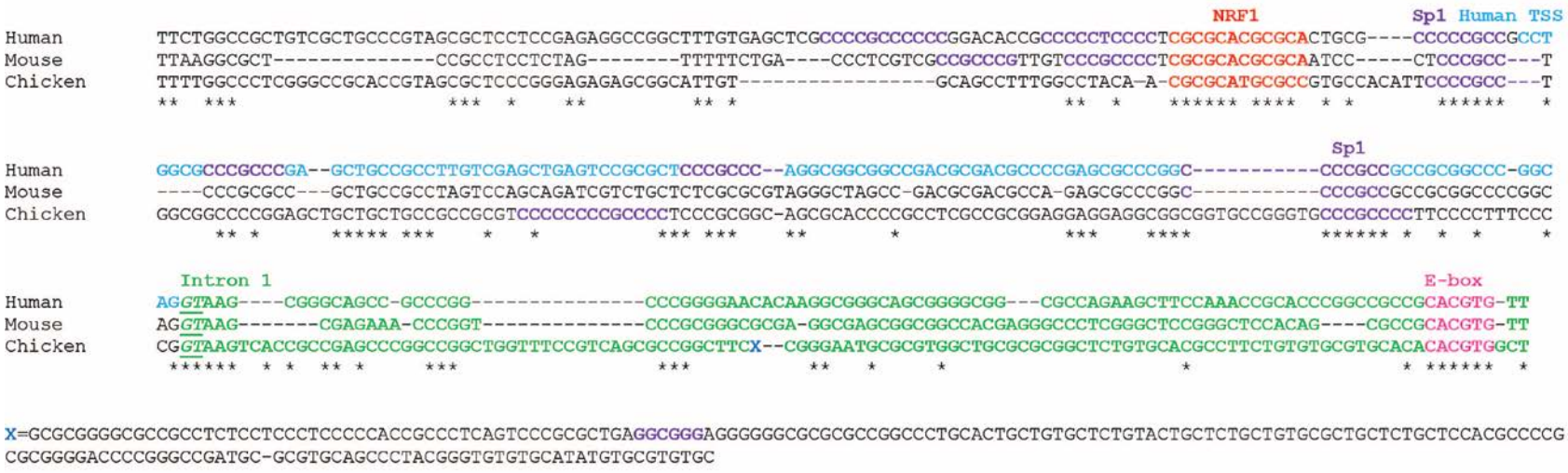
(b) *PPP5C* promoter (ChIP)

(c) *FBXL15* promoter (ChIP)


Cat
Dog
Horse
Cow
Human
Mouse
Sp1

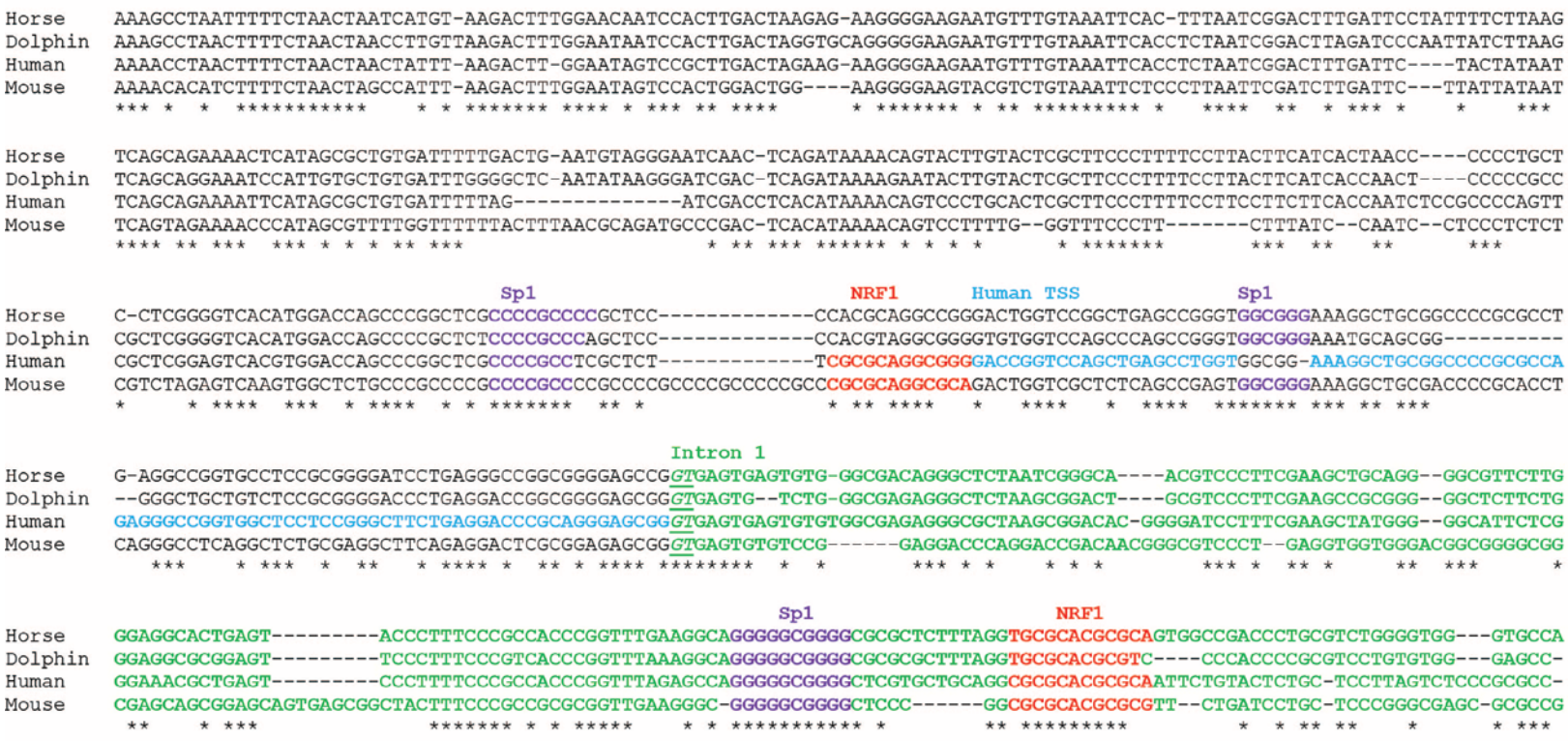

Cat
Dog
Horse
Cow
Human
Mouse
Sp1
NRF1
Exon 1 coding sequence
Intron 1
Sp-fam

(i) *WDR5* promoter (ChIP)

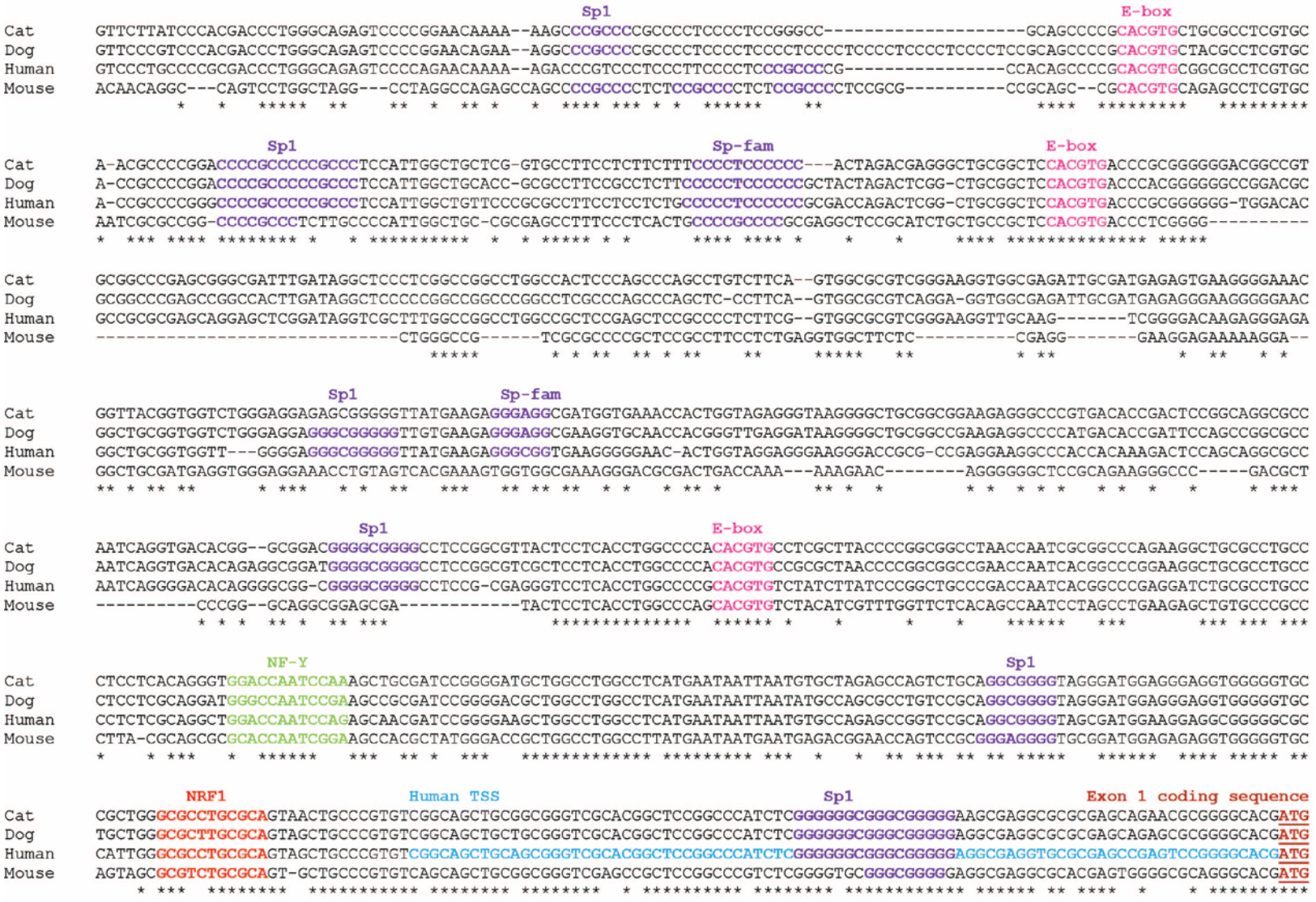


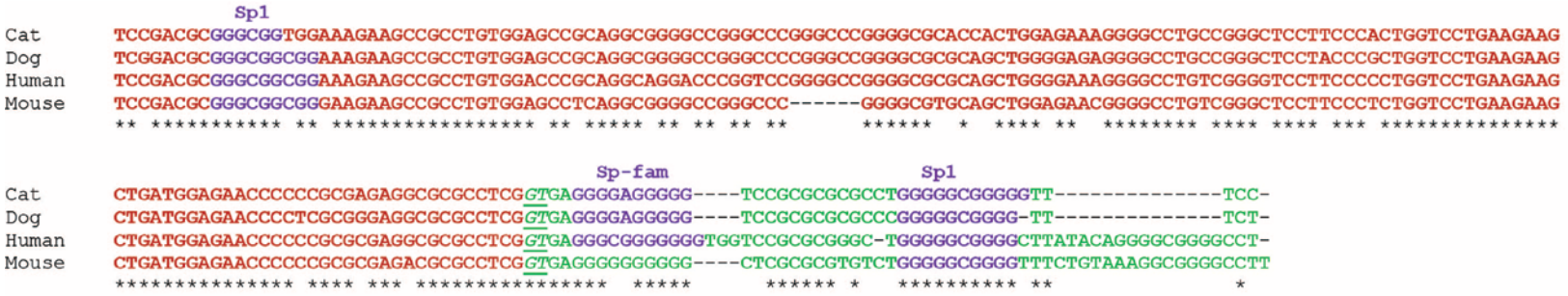
X=GCGCGGGGCGCCGCCTCTCCTCCCTCCCCCACCGCCCTCAGTCCCGCGCTGAGGCGGGAGGGGGGCGCGCGCCGGCCCTGCACTGCTGTGCTCTGTACTGCTCTGCTGTGCGCTGCTCTGCTCCACGCCCCG
CGCGGGGACCCCGGGCCGATGC-GCGTGCAGCCCTACGGGTGTGTGCATATGTGCGTGTGC

(e) *TIMELESS* intron 1 (ChIP)

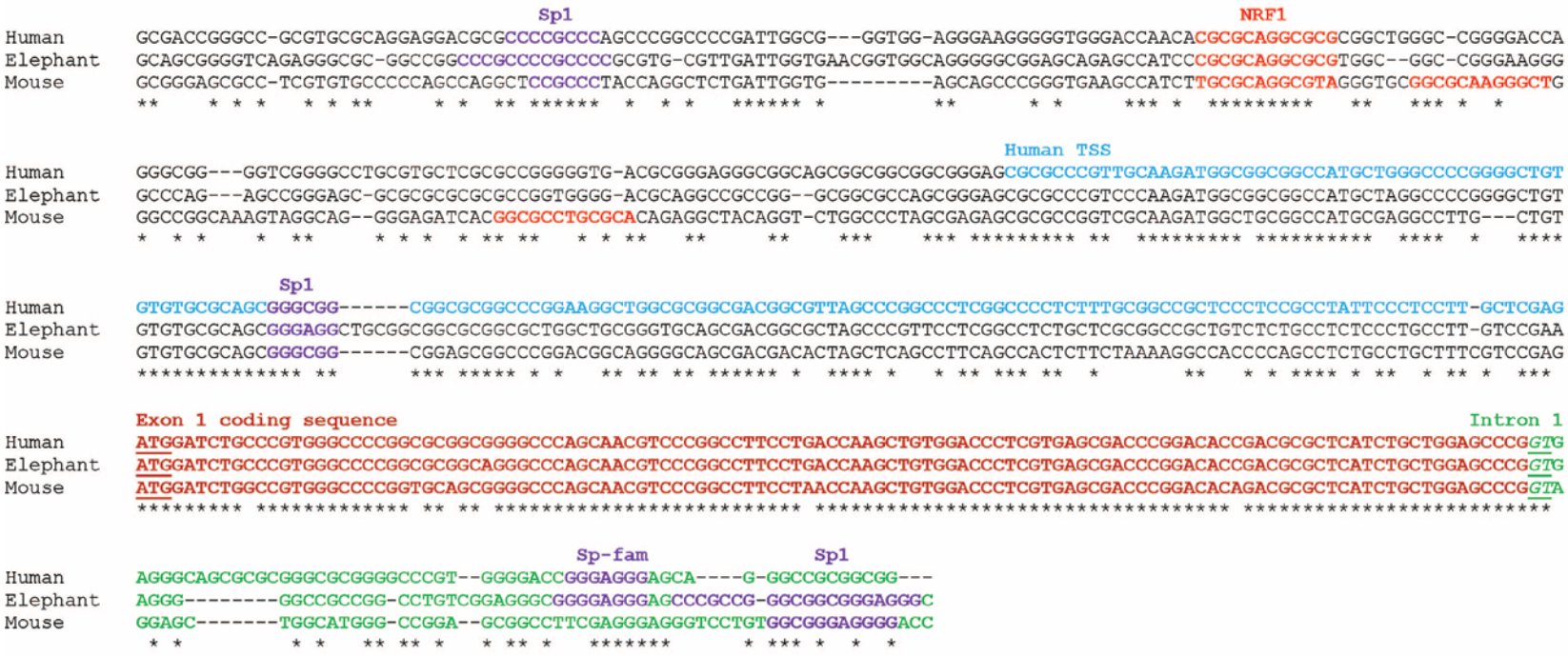


(f) *TEF* promoter (ChIP)

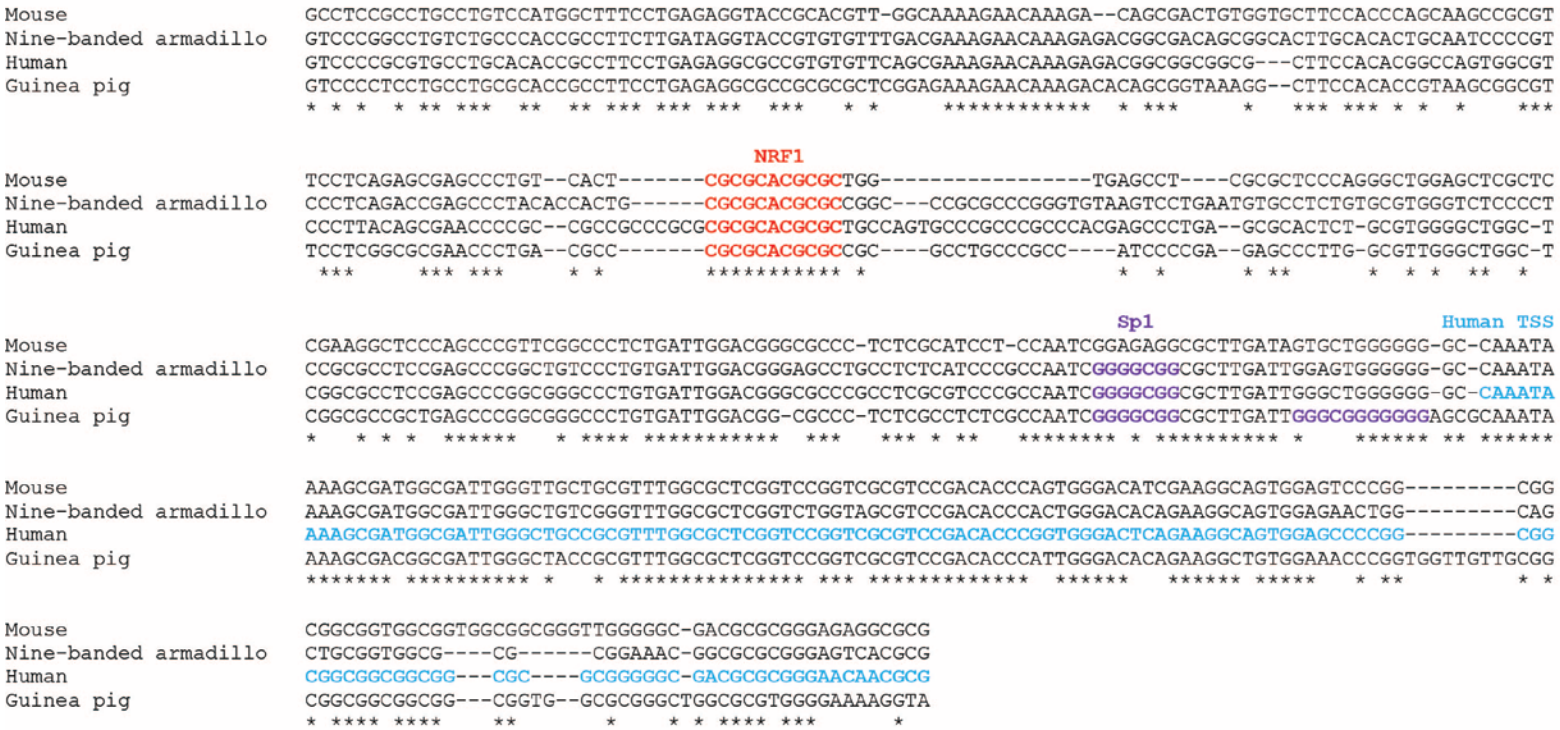




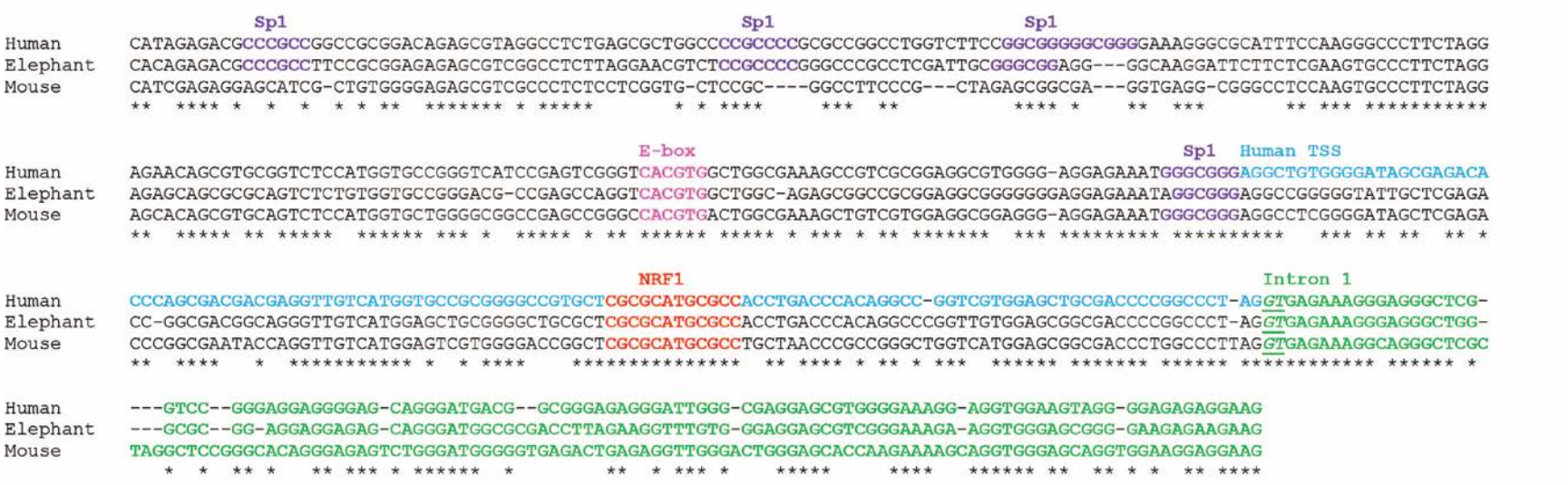
(g) *HSF1* promoter (ChIP)



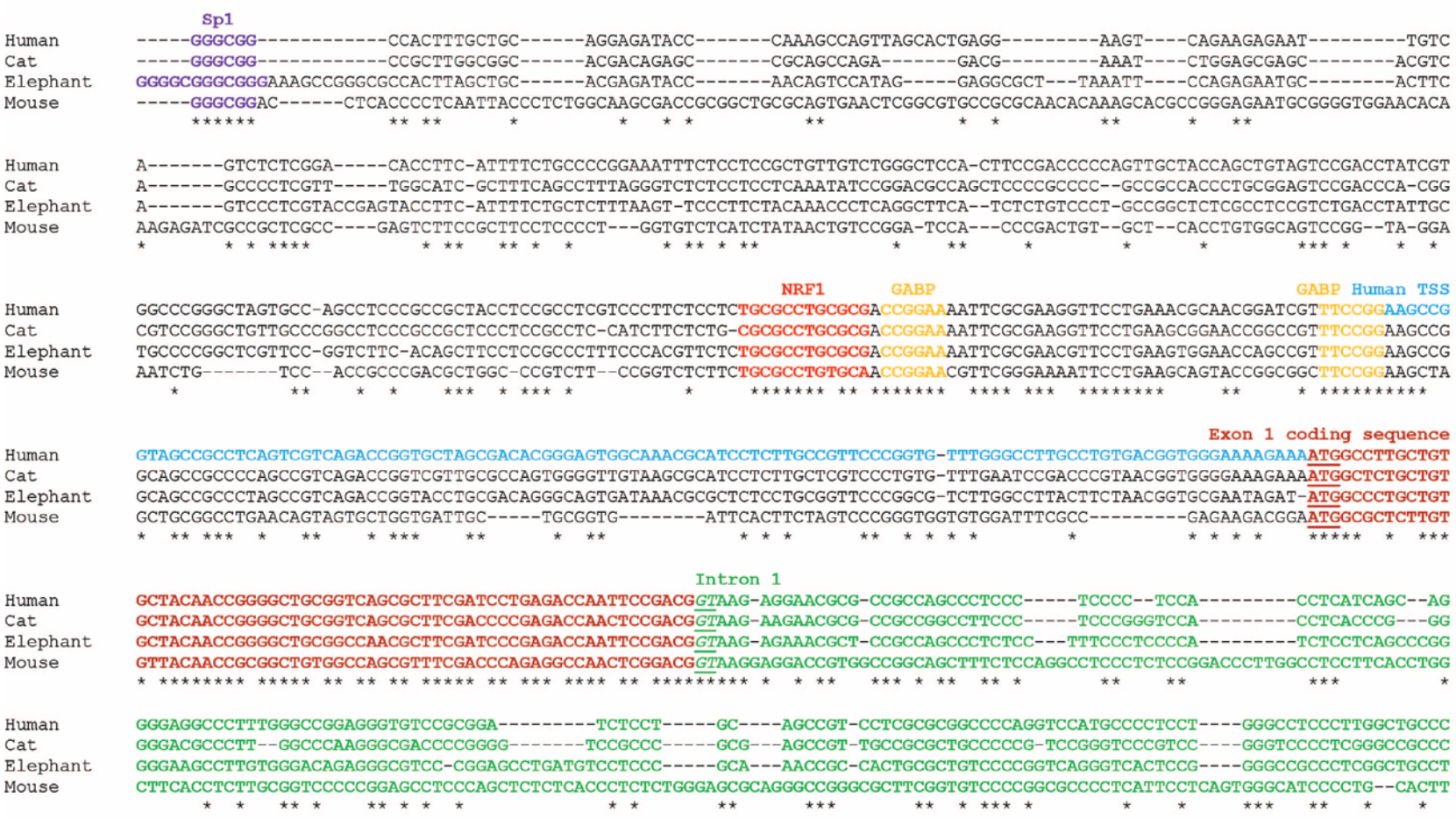
(h) *EZH2* promoter (ChIP)



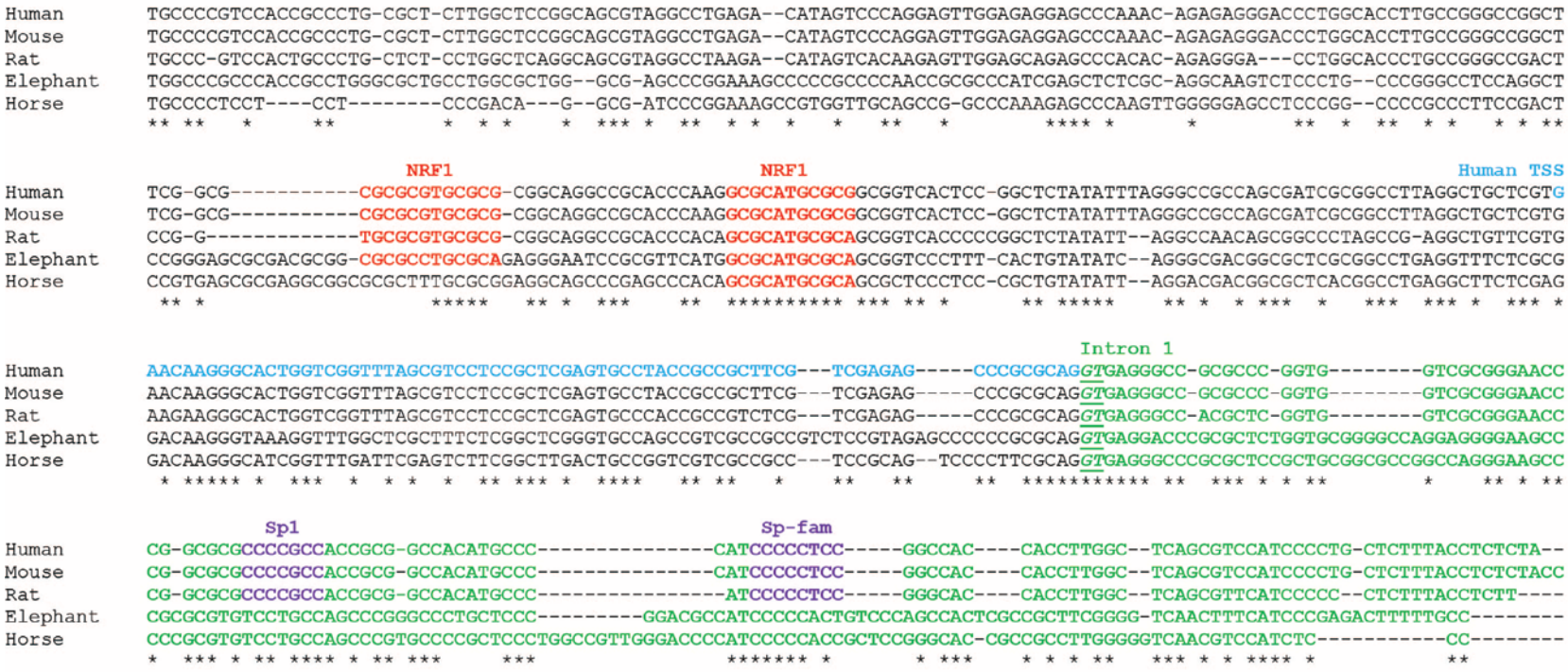
(i) *CIPC* promoter (ChIP)



(j) *CHORDC1* promoter (ChIP)

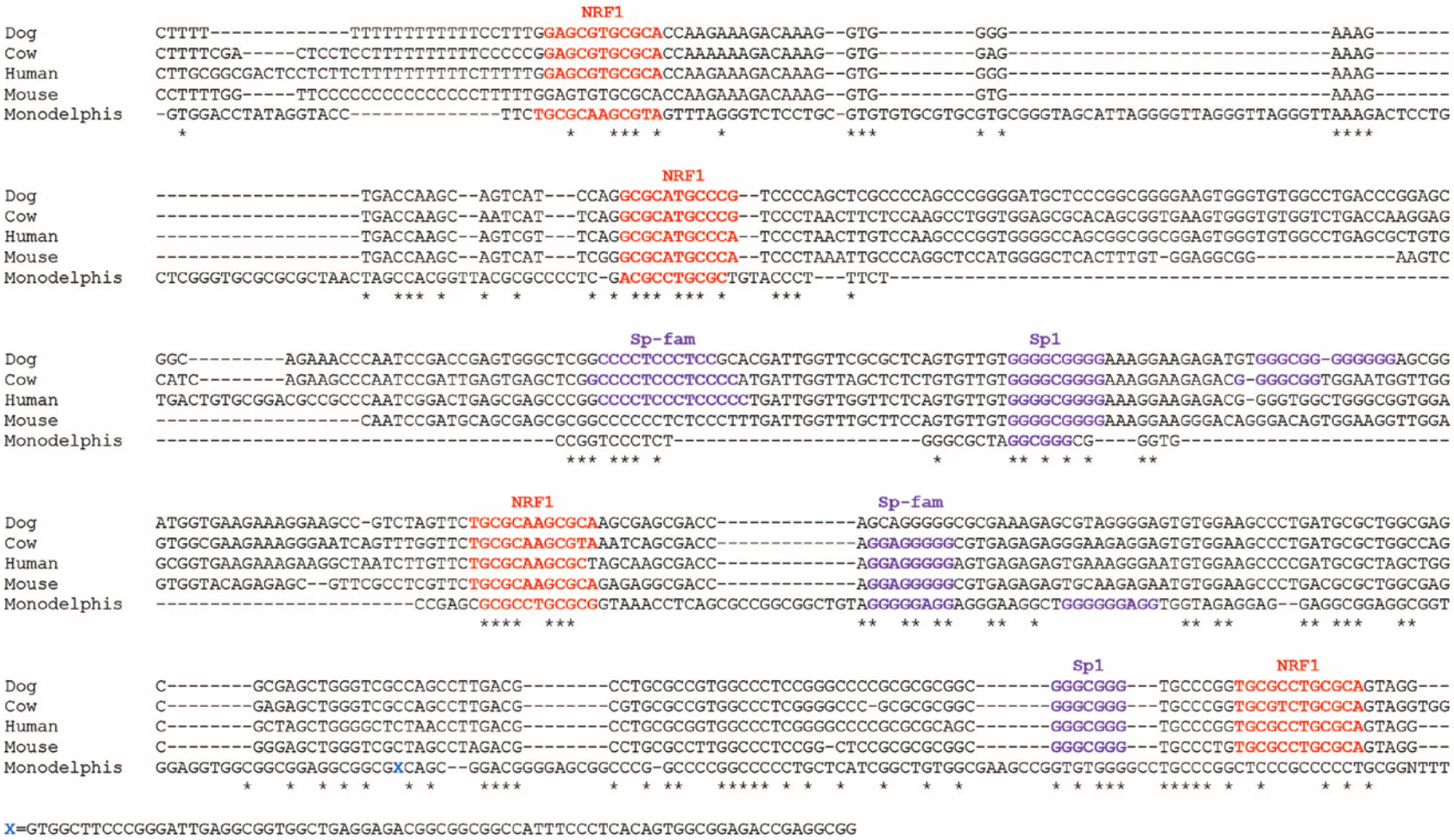


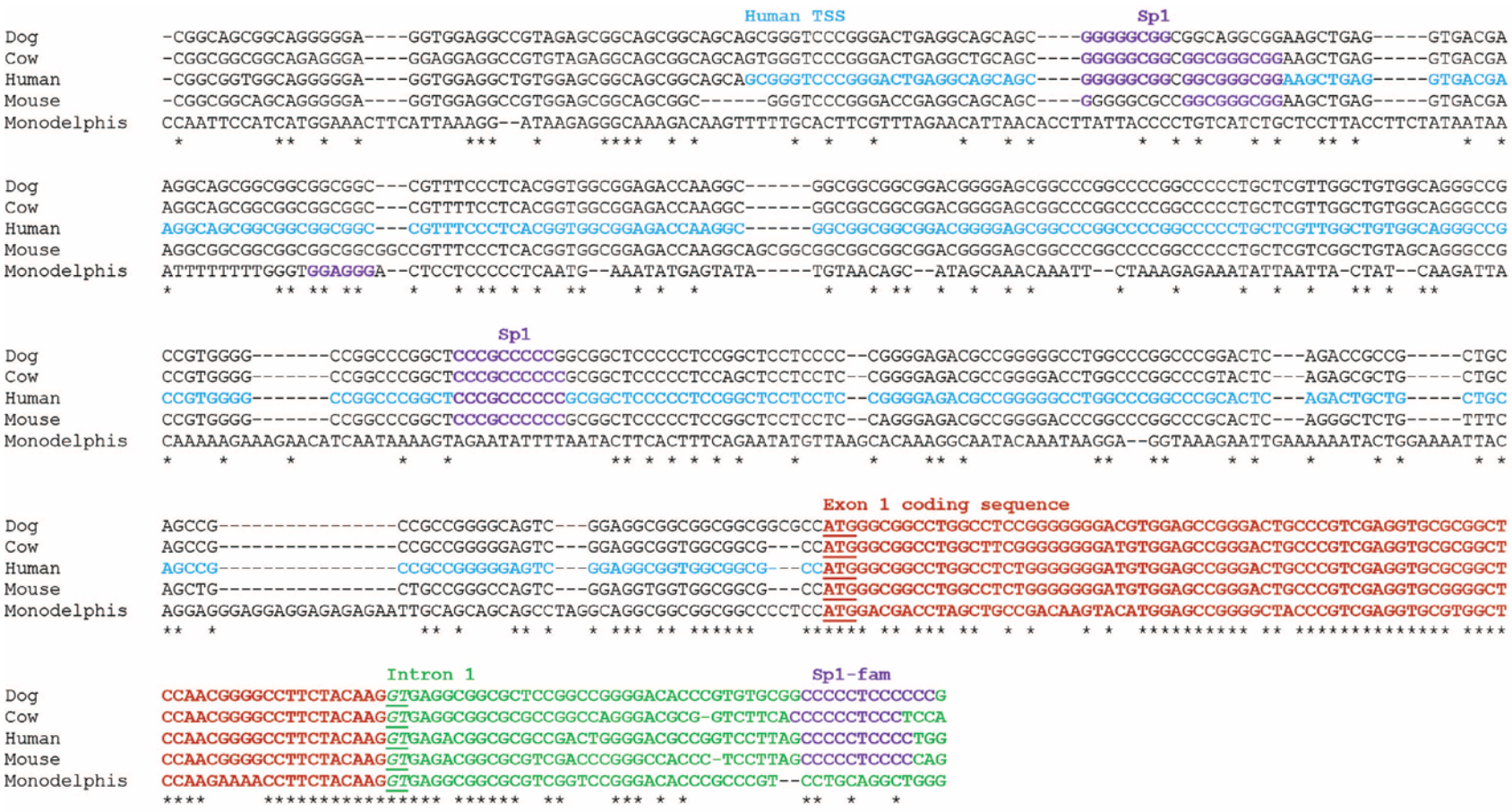
(k) *ALAS1* promoter (ChIP, pub)



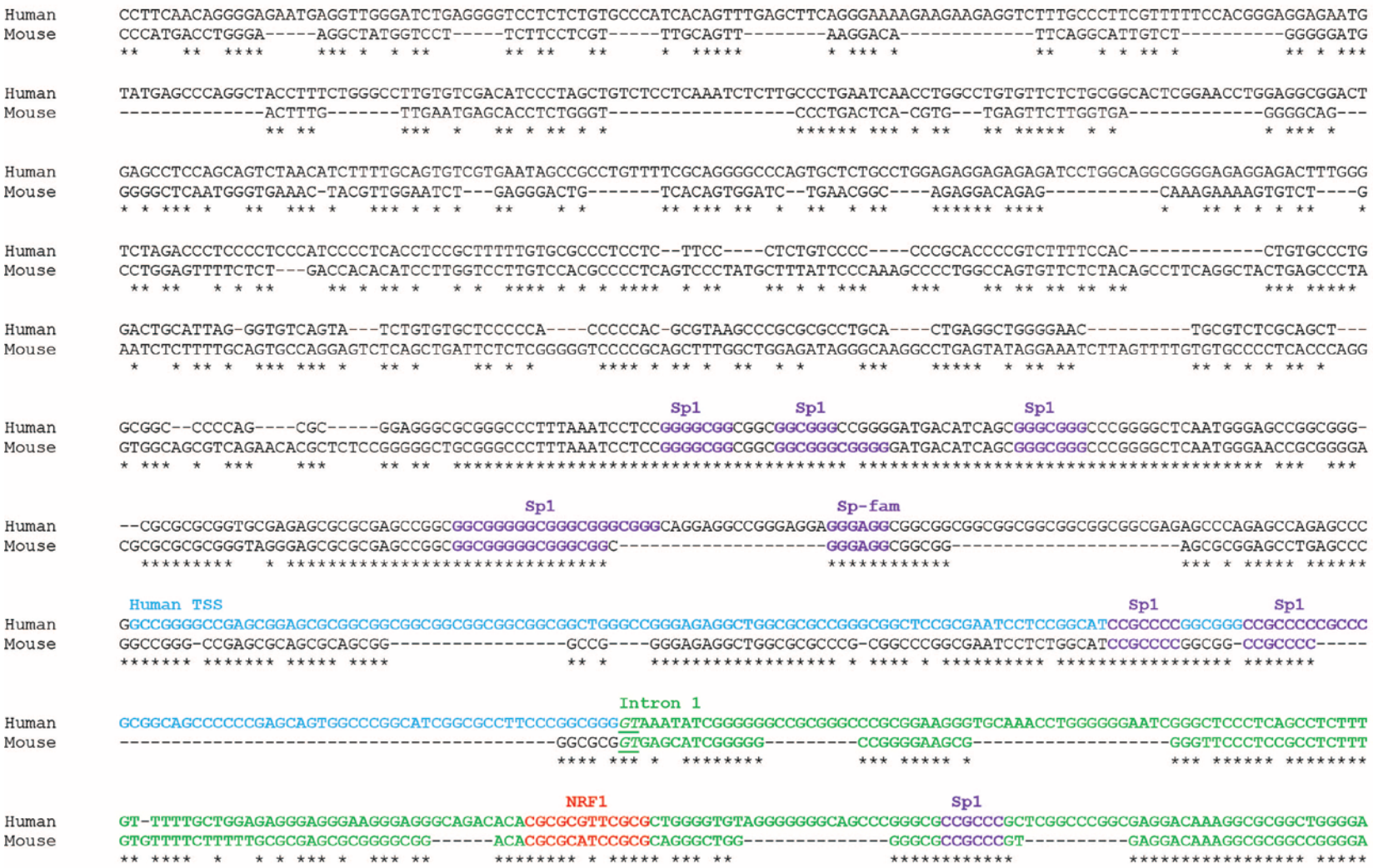
(l) *FMR1* promoter (pub)

(m) *FXR2* promoter (pub)

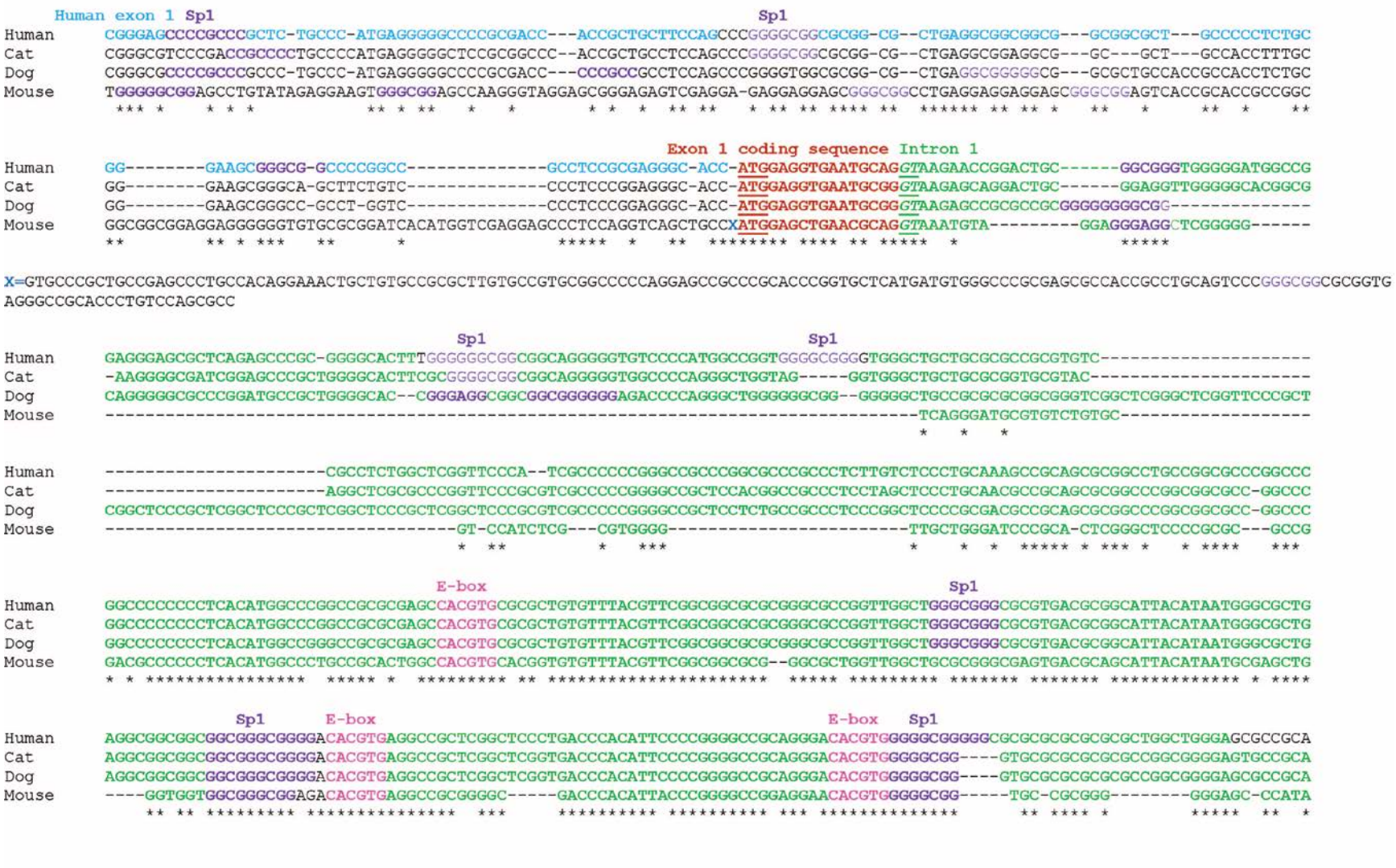


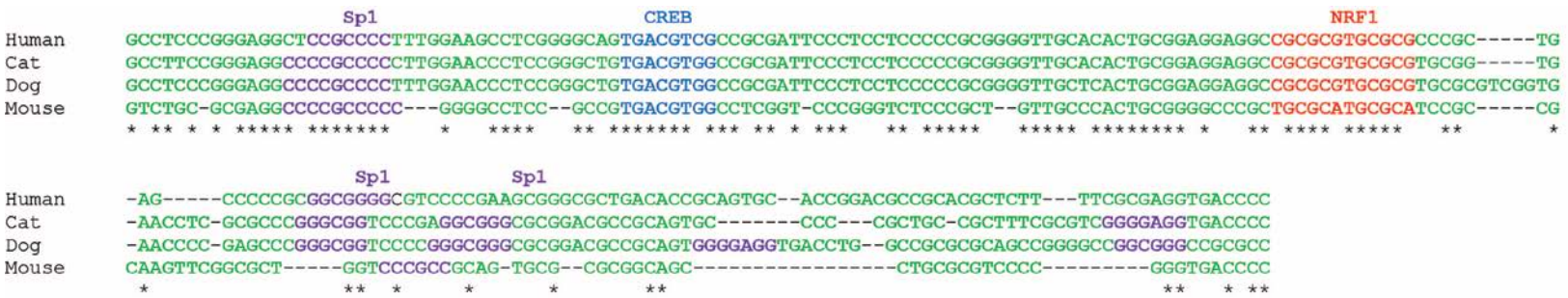

Human TSS
Sp1
Dog
Cow
Human
Mouse
Monodelphis
Sp1
Exon 1 coding sequence
Intron 1
Sp1-fam

(n) *CSNK1E* intron 1 (no exp)



(o) *NR1D2* intron 1 (no exp)



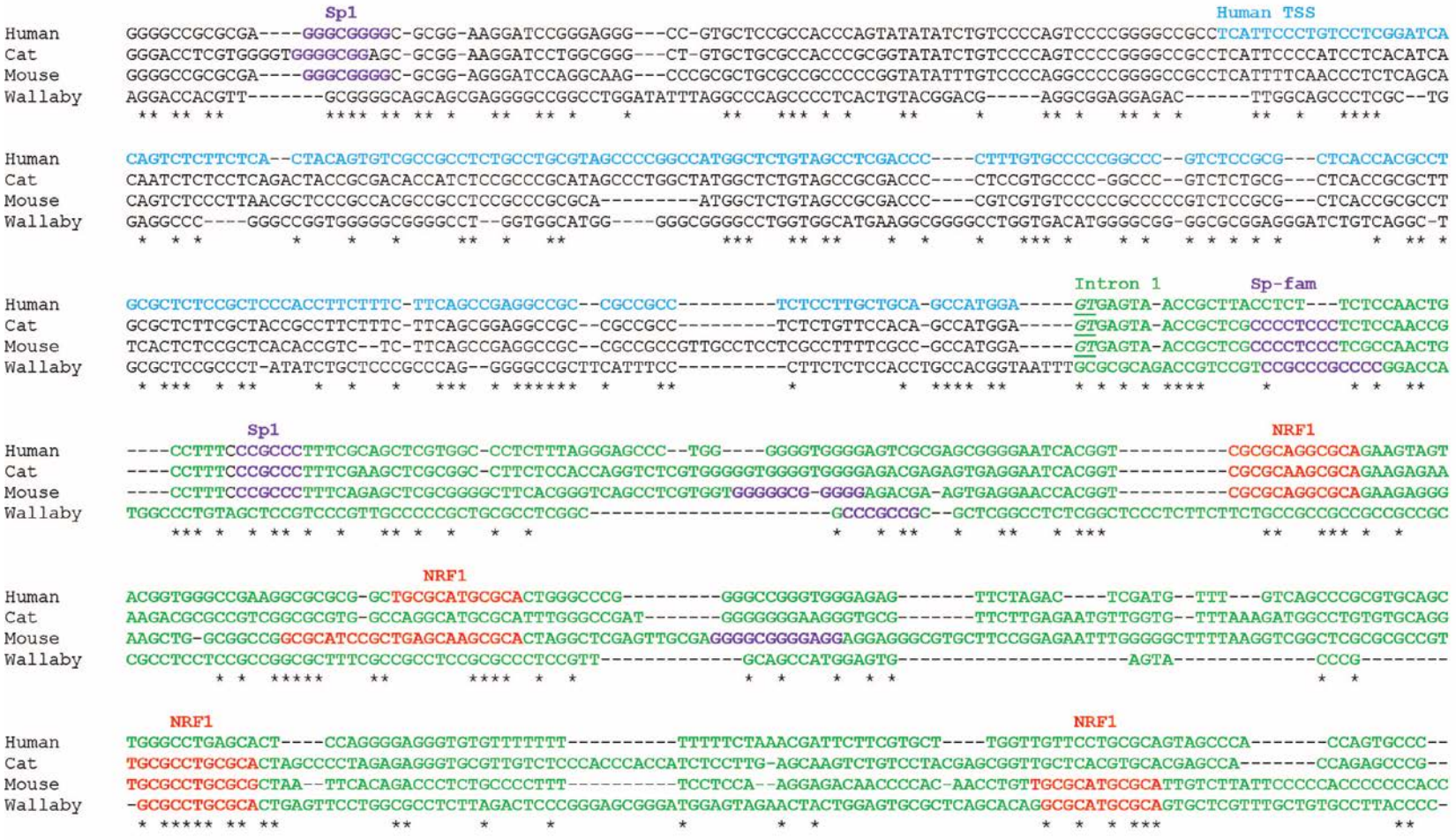


(p) *CREM* promoter (no exp)

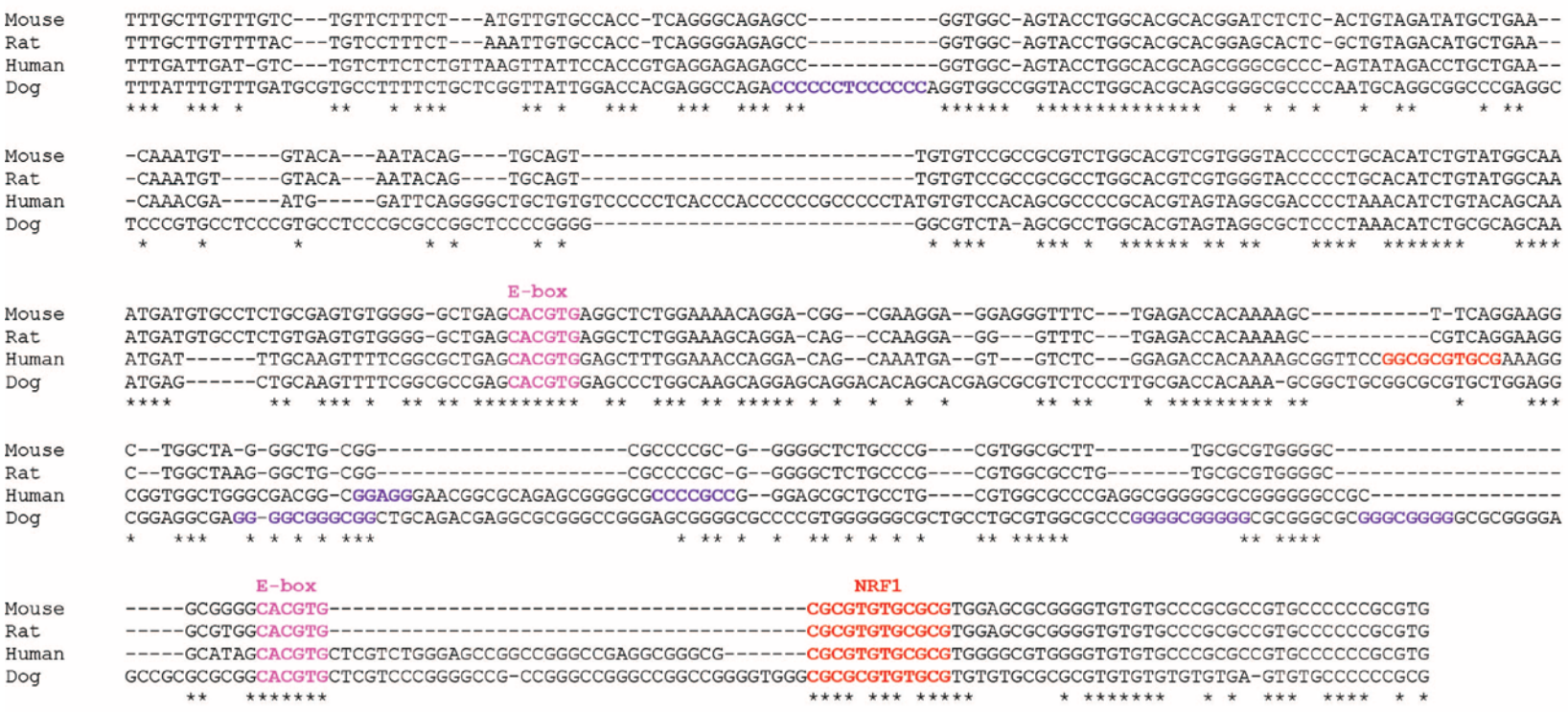
(q) *MYBBP1A* promoter (no exp)

(r) *RAB3A* intron 1 (no exp)

(s) *ATF5* intron 1 (no exp)



(t) *PROK2* promoter (no exp)



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